ROLE OF SMALL NON CODING RNAs IN GENE REGULATION IN *Escherichia coli*

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Mahima Pandor

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List of Abbreviations

Ap	Ampicillin
APS	Ammonium persulphate
Ag43	Antigen 43
BLAST	Basic Local Alignment Search Tool
bp	base pair (s)
DNA	Deoxyribonucleic acid
dNTPs	2-deoxy-5'-triphosphates
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immuno-sorbent analysis
EtBr	Ethidium bromide
FACS	Fluorescence Activated cell sorting
FBS	Fetal Bovine Serum
Fig.	Figure
FITC	Fluorescein Isothiocynate
GFP	Green Fluorescent Protein
IPTG	Isopropyl β-D thio galactopyranoside
Kbp	Kilo base pair
Km	Kanamycin
LB	Luria broth
MCS	multiple cloning site
MW	molecular weight
mRNA	messenger ribonucleic acid
NCBI	National Centre for Biotechnology Information
ncRNA	Non coding RNA
nt	Nucleotides
OD_x	Optical Density at wavelength of x nm
ONPG	ortho-Nitrophenyl-ß-D-galactopyranoside
ORF	open reading frame
Ori	Origin of replication
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PBS	Phosphate Buffered Saline
PQ	Paraquat
qRT-PCR	Quantitative Real time PCR
RBS	Ribosome binding site
RE	Restriction Endonuclease
RO	Reverse Osmosis purified
rpm	revolutions per minute

RT	Room temperature
RT-PCR	Reverse Transcription PCR
SD	Shine Dalgarno sequence
sRNA	Small RNA
TBE	Tris-Borate EDTA
TEMED	N,N,N`,N`-Tetramethylen ethylendiamide
Tet	Tetracycline
Tris	Tris (hydroxymethyl) aminomethane
UV	Ultravoilet
v/v	Volume per volume
w/v	Weight per volume
X-Gal	5-Bromo-4-chloro-3- indolyl-β-D galactopyranoside
k	kilo (10^3)
m	milli (10^{-3})
µ	micro (10^{-6})
n	nano (10^{-9})
˚C	degree Celsius
Da	Dalton
g	gram
h	hour
l	litre
M	molar
min	minute(s)
s	second
U	Unit (enzyme activity)
V	Volt

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Abstract

The *E. coli* sRNA, RyjA (~140 nucleotide), with three distinct stem loop regions is expressed in stationary phase and transcribed convergently opposite to the neighbour, *soxR,* the key regulator of oxidative stress management regulon *soxR*S. The complementary overlap in the transcription terminator at the 3' end of *ryjA* to that of *soxR* suggested RyjA to be a *cis*-encoded regulatory sRNA for *soxR* expression.

RyjA was cloned, overexpressed as well as disrupted. Strains were also constructed containing RyjA modified to delete or substitute individual stem loop. The substitution of stem 3 region of RyjA carried a modified transcription terminator that had no complementarity to the 3' end of *soxR* but maintained the identical secondary structure.

The overexpression of RyjA under oxidative stress, induced by paraquat resulted in reduction of cell growth, SOD activity and increased sensitivity to higher doses of ampicillin as compared to the vector control. This was paralleled by a reduction in the expression of *soxR::lacZ* and *soxS::lacZ* fusions. Real time PCR also revealed a decrease in the level of transcripts of *soxR* (~0.6 fold)*, soxS, sodA* and *nfo,* all associated with the oxidative stress pathway. Conversely the disruption mutants of chromosomal copy of *ryjA* exhibited under oxidative stress increased growth rate and increased levels of *soxR* (2.4 fold) and the other genes of SoxRS regulon as measured by real time PCR. .

The presence of stem 3 mutant derivatives in multicopies indicated increased levels of transcripts of SoxRS regulon. This observation suggested that RyjA modified derivatives either competitively inhibited the function of the wild type transcript by titrating out a common mediator required for its regulatory role or by positive modulation arising from excess of stem loop 1 or 2. The oxidative stress also resulted in a decrease in the levels of RyjA transcripts. All the above observations collectively suggest that RyjA probably mediates regulation of SoxR by 3' end base pairing and prevent the futile expression of SoxR in the absence of oxidative stress

Another small RNA IsrC (204 nt) situated at 41.4 minutes on the *E.coli* genome, lies in the 5' regulatory region of *flu* gene encoding Antigen 43, an auto transporter outer membrane surface protein. The *isrC* and *flu* genes are part of the cryptic prophage CP4-44. The isrC was overexpressed from the *lac* promoter in a multicopy plasmid. A 1.6 fold increase in the expression of Antigen 43 was observed in real time PCR under the influence of overexpression of *isrC*. The highest titre for the antibody against Antigen 43 protein was detected in IsrC overexpressing strain in comparison to the control strain and a uropathogenic *E. coli* strain. Thus IsrC appears to be the probable positive regulator of Antigen 43 expression. The increased expression of Antigen 43 was however not reflected in strong autoaggregation or enhanced uptake of the cells by macrophages as several proteins are capable of conferring autoaggregation phenotype and uptake by macrophages.

Review of Literature

1.1. Regulation of Gene Expression

Regulation of gene expression is essential in all organisms so that the genes are expressed at the right time and in correct levels to maintain the cell or promote growth and proliferation. The regulation of gene expression can occur at any step, from DNA to RNA transcription to the post translational modification of a protein. One of the early discovered examples of the gene regulation system is that of the *lac operon*, observed by Jacques Monod in which proteins involved in lactose metabolism are expressed by *E. coli* only in the presence of lactose and absence of glucose (Jacob and Monod, 1961). The gene expression in cells can be at the transcriptional level or translational level. There are sublevels within each level and are described below in brief.

1.1.1. Regulation of transcription

Transcriptional initiation is regulated by a combination of factors, including DNA sequence and its three-dimensional topology, proteins and small molecules. Regulation of initiation begins with the prudent distribution of RNA polymerases (RNAP) between the competing promoters. The σ factor which is component of RNAP holoenzyme recognizes the core promoter elements and position the RNAP at the target promoter (Wösten, 1998). Distinct sigma factors compete with for binding to a common pool of RNA polymerases, thus achieving condition-dependent differential expression. Another important class of bacterial regulators is transcription factors that bind to specific sites in the promoter region. As an outcome of interactions or due to steric interference between the transcription factors and RNA polymerases, transcription activation or repression occurs (Bintu *et al*., 2005). Some bacterial gene regulatory protein can recognizes specific sites on DNA by a helix-turn-helix motif and act as activators or repressors depending upon their precise placement of the binding sites in DNA (Alberts, 2008).

Elongation and termination of transcription regulation involves anti termination and attenuation. In anti termination the RNAP is modified to a termination resistant state, usually by association with one or more protein factors. Attenuation on the other

hand regulates the formation of a Rho-independent terminator structure in the leader region of the operon leading to the termination of transcription (Alberts, 2008).

1.1.2. Regulation of translation

Prokaryotic translational initiation involves binding of small ribosomal subunit to mRNA aided by three initiation factors (IFs). IF2 binds tRNA (initiator formylmethionyl-tRNA) and adjust it in the P site, while IF1 stimulates IF2 activity and IF3 controls the accuracy of codon-anticodon recognition. The size and optimum spacing of the initiator element of translation i.e. Shine-Dalgarno (SD) sequences and the start codon are crucial for translational initiation regulation (reviewed by Kozak, 2005).

The expression of prokaryotic genes through polycistronic transcripts is a mode of translational regulation wherein the translation of downstream cistron is coupled to the preceding cistron. Translational regulation involves *cis*-acting mRNA sequences that form secondary or tertiary structures sequestering the ribosome binding site (RBS). The binding of *trans*-acting proteins and/or non coding antisense RNA molecules can allosterically control alternative structures within the same mRNA leader sequences or sterically impede ribosomal entry.

The regulation of translational elongation involves frameshifting and pausing of the ribosome. The frameshifting is triggered by two elements, a slippery sequence in the mRNA, where tRNA movement or misalignment is favoured and a stimulator that enhances the process, probably by induction of ribosomal pause (Namy *et al*., 2004).

The special case of pausing of ribosome also called as elongation arrest is brought about by the Signal Recognition Particle (SRP). The SRP binds tightly to ribosome-nascent chain complexes and results in elongation arrest followed by docking the ribosome on to the translocation apparatus and establishment of co-translational translocation (Walter and Blobel, 1981).

1.2. RNA as a regulatory molecule

RNA is categorized in three major classes with distinct cellular roles: mRNAs- the genetic material of some viruses, and function in genetic information transfer; ribosomal RNAs and tRNA adaptors, involved in protein synthesis.

Within recent years, however, several new functions of RNAs surfaced globally, which could be enzymatic or regulatory such as the eukaryotic snRNA (snoRNA-splicing), telomerase RNA (telomerase maintenance), siRNA (RNA silencing), pri-miRNA, miRNA (micro RNA- regulation), gRNA (RNA editing), ribozymes, piwi-interacting RNAs (gametogenesis) and the sRNAs in bacteria.

1.3. Evolution of RNA as regulators

The regulatory non coding RNAs were identified few decades ago in both prokaryotes and eukaryotes. In 1961, Francois Jacob and Jacques Monod put forward the hypothesis that regulatory genes could produce RNA molecules that would interact with operators by base pairing, either at the transcriptional level or the post transcriptional level (Jacob and Monod, 1961). A similar proposal was made a few years later by Britten & Davidson (1969) to explain the eukaryotic gene regulation. These views were quickly abandoned after the discovery that protein complexes were involved in the control of almost every step of gene expression.

The direct postulation of an antisense mechanism of action by Cro RNA was discovered more than 40 years ago in λ phage (Spiegelman *et al*., 1972). Later in 1981 it was found that two antisense RNAs, RNAI and CopA, controlled the replication and copy number in ColEI and R1 plasmids respectively by base pairing with the RNA that is cleaved to produce the replication primer (Tomizawa *et al*., 1981; Stougaard *et al*., 1981).

This finding was followed by the discovery of another small RNA (70 nucleotide) that is transcribed from the pOUT promoter of the Tn10 transposon and represses transposition by preventing translation of the transposase mRNA (Simons and Kleckner, 1983).

However in eukaryotes, setting aside the already known snoRNAs, tRNAs and rRNA, the first regulatory non coding RNA (ncRNA) described in literature was *H19*, a large RNA maternally expressed in mice (Brannan *et al*., 1990; Bartolomei *et al*., 1991). Though the *H19* gene was identified and cloned some 20 years ago, the exact function of this long ncRNA was not known until recently. *H19* gene modulates the expression of several genes of imprinted gene network (IGN) in the mouse embryo (Gabory *et al*., 2009). The next RNA regulator, *lin-4* (~21 nt) was identified in 1993

while studying the *C.elegans* larval development. The *lin-4* RNA pairs with the 3' UTR of *lin-14* mRNA and represses its translation. The *lin-14* mRNA encodes protein which triggers transitions from cell divisions of the first larval stage to those of the second (Lee and Ambros, 2001). Another regulatory RNA, *let-7* discovered again in *C.elegans* encodes a 22nt regulatory RNA which promotes the transition from late larval to adult fates similar to *lin-4* mode of action (Reinhart *et al*., 2000; Slack *et al*., 2000). Around the same time RNA interference (RNAi) which involves silencing of the gene expression by the exogenous double stranded RNA which base pairs with the transcript and degrades was discovered. By the end of the century and with the advent of the sequencing of genomes of various organisms, the acceptance and appreciation for non coding RNAs as regulators of gene expression increased. More than hundred non coding RNAs were discovered in flies, worms, plants and human cells. The first Nobel Prize went to Andrew Fire and Craig Mello in 2006 for the discovery of antisense interference in eukaryotic systems.

1.4. RNA world

The catalytic and regulatory properties of RNA as discussed above suggests that a world filled with RNA based life preceded the current DNA based life where RNA could both, store information and acts as an enzyme. The phrase "RNA world" was coined by Walter Gilbert in 1986 and refers to a hypothetical stage in the origin of life on earth where RNA carried out both, task of storing genetic information and catalytic roles necessary in a very primitive self replicating system (Gilbert, 1986). The four processes of RNA chemistry- replication, cellularization, translation and metabolism- account for the explanation of evolution in RNA world. The other discoveries demonstrating the role of RNA molecule in RNA splicing, RNA editing, telomerase maintenance, germline development, regulation of gene expression etc. further assert the concept of RNA world hypothesis (Eddy, 2001; Vlassov *et al*., 2005).

RNAs function as a regulators of gene expression in both prokaryotes and eukaryotes and modulate the expression of target mRNA in a variety of ways.

1.5. Non coding RNAs in Eukaryotes

The ncRNAs in eukaryotes include introns and independently transcribed RNAs. ncRNAs control various levels of gene expression in physiology and development,

including transcription, translation, RNA splicing, editing, silencing and turnover, chromatin architecture and epigenetic memory (Mattick and Makunin, 2006). The major groups of ncRNAS successfully studied in recent years are discussed below in brief, in particular, ribozymes which regulate mRNA expression by associating with ribonucleoprotein complexes (RNPs), snRNAs and snoRNAs involved in RNA modification and miRNAs, siRNAs and piRNAs responsible for gene silencing.

1.5.1. ncRNAs involved in post transcriptional gene splicing

1.5.1.1. Ribozymes

Ribozymes (ribonucleic acid enzymes) are catalytic RNA molecules, catalyzing the cleavage or ligation of the RNA phosphodiester backbone and include the self-cleaving ribozymes and the self-splicing ribozymes. The self-cleaving ribozymes catalyze the cleavage of their own phosphodiester bonds while self-splicing ribozymes catalyze both, the cleavage and ligation of the phosphodiester bonds.

The self-cleaving ribozymes (40-200 nt long) have various secondary structures and three dimensional fold and catalyze sequence-specific cleavage and ligation. The small self-cleaving ribozymes include the hammerheads, hairpins, hepatitis delta virus (HDV), Varkud satellite (VS) and *glmS* ribozymes (Ferre-D'Amare and Scott, 2010). Out of these, the *glmS* ribozyme in gram positive bacteria (such as *Bacillus subtilis*) also functions as a key riboswitch component. The metabolite responsive self-cleaving *glmS* ribozyme is located in the 5' region of bacterial *glmS* gene that encodes glucosamine-6-phosphate (GlcN6P) synthase. It forms a conserved catalytic core which binds to specific cofactor, GlcN6P that promotes ribozyme self-cleavage and results in down regulation of *glmS* gene expression (reviewed in Talini *et al*., 2009). As the *glmS* ribozyme permits the feedback regulation of GlcN6P levels, it also functions as a riboswitch.

RNase P is the only known naturally occurring cleaving ribozyme identified to catalyze the RNA cleavage reactions in *trans*, involving multiple substrate molecules. The RNase P ribozyme removes extra sequences from 5' end of pre-tRNAs and few other RNAs. Recent findings suggest that human nuclear RNase P is required for efficient transcription of various RNA polymerase III transcribed small non coding

RNA genes, such as tRNA, 5S rRNA, SRP RNA and U6 snRNA (Reiner *et al*., 2006).

The splicing ribozymes are large RNAs involved in the excision of introns and linking the boundary exon from precursor RNAs (pre-RNAs) and require assembly of RNA-protein complexes (Serganov and Patel, 2007). The splicing ribozymes include two classes of heterogeneous self-splicing introns (groups I and II), which can be found in many tRNA, mRNA and rRNA precursors. For splicing, the location of splice sites are defined by the interactions between the 5' region of the intron and two exons (domains P1 and P10) in group I introns, and by two or three pairs of interactions between intron binding sites (IBSs) and exon binding sites (EBSs) in group II introns.

1.5.1.2. Small nuclear and nucleolar RNAs (snRNAs & snoRNAs)

snRNAs are abundant, non coding, small RNA molecules found in the nucleus of eukaryotic cells. They are generally 100-300 nucleotides long and are involved in RNA splicing, regulation of transcription factors and telomere maintenance. The snRNAs are always associated with specific proteins and form small ribonucleoprotein complexes (snRNPs). These large RNA-protein complexes, snRNPs, consist of U1, U2, U4, U5 and U6 snRNAs and form a spliceosome complex which catalyzes splicing. The U-snRNAs utilize base pairing interaction to identify pre-mRNA splice sites (intron-exon junctions) and serve as catalytic centre within the spliceosome (Hopper, 2006; Matera *et al*., 2007).

The snoRNAs (small nucleolar RNA) comprise of two families, the C/D box snoRNAs and H/ACA snoRNAs. The C/D and H/ACA snoRNAs use base complementaries to guide 2'-O-ribose methylation and pseudouridylation in rRNAs, (conversion of uridine to pseudouridine) respectively. The C/D and H/ACA RNAs function in parallel to both process and modify key ribosomal rRNA regions (for example, the peptidyl transferase centre and the mRNA decoding centre) which are essential for ribosomal functions. Other modification targets include snRNAs in eukaryotes, transfer RNAs in archaea, spliced leader RNAs in trypanosomes and at least one specific mRNA in mammals (Eliceiri, 2006; Rogelj, 2006).

1.5.1.3. RNA editing

RNA editing causes site specific alterations in RNA sequence of tRNA, rRNA and mRNA and has been observed in all organisms from protozoa to viruses, plants and animals. It includes nucleoside modifications such as cytidine (C) to uridine (U) and adenosine to inosine (I) deaminations and non-templated nucleotide additions and insertions. RNA editing events create new open reading frames or result in codon changes to restore highly conserved amino acid residues. In addition the single base modifications can generate multiple protein isoforms with distinct biological functions, affect splicing patterns or alter the structure/function of non coding RNA species (Gott and Emeson, 2000). RNA editing involving insertion or deletion or conversion of bases is discussed in brief:

(i) Editing by insertion or deletion: RNA editing through insertion or deletion of uracil residue was first observed in mitochondrial RNA of kinetoplastid protozoa (Benne *et al*., 1986). The pre-edited RNA base pairs with guide RNA (gRNA) and this double stranded duplex is enveloped by large multi protein complex, editosome, which catalyzes editing.

(ii) Editing by deamination: C-U and A-I editing: The cytidine and adenosine demainase deaminates a cytidine base into a uridine base and an adenosine base into an inosine base. For example, the C-U editing was first observed in apolipoprotein B (*apoB*) in humans in which a glutamine codon (CAA) is changed to a stop codon (UAA) (reviewed in Gott and Emeson, 2000).

1.5.2. Non coding RNAs involved in gene silencing

1.5.2.1. MicroRNAs (miRNAs)

MicroRNAs (miRNAs), post transcriptional regulators, are endogenous ~22-nucleotide long RNA sequences that bind to complementary sequences in the 3' UTR of multiple target mRNAs. This results in down-regulation of target expression, at either the transcript or the translational level. They are found in worms, flies, plants and vertebrates. Most of the miRNAs are derived from independent transcription units and are located in intergenic regions or in antisense orientation to the genes which they regulate while a minority of them are located in the introns of pre-mRNA (Lau *et al*., 2001; Lee *et al*., 2004; Lagos-Quintana *et al*., 2001; Lee *et al*., 2001).

miRNAs appear to modulate majority of physiological processes; cell differentiation, proliferation and survival. The aberrant and altered expression of miRNAs and the deregulation of genes controlled by miRNAs have been implicated in various pathological disorders including cancer, cardiovascular, metabolic and neurodegenerative diseases (Shukla *et al*., 2011).

The biogenesis of miRNA: The miRNA genes are predominantly transcribed by RNA polymerase II (PolII) to generate a stem loop containing primary miRNA (pri-miRNA) of sizes ranging from hundreds to thousands of nucleotides (Lee *et al*., 2004; Cai *et al*., 2004). The pri-miRNA, in the nucleus is cleaved by Microprocessor, a multiprotein complex to produce ~70 nt hairpin precursor miRNA (pre-miRNA). The core components of microprocessor complex include RNAase III enzyme Drosha and double stranded RNA binding domain (dsRBD) protein DGCR8/Pasha (Carthew and Sontheimer, 2009).

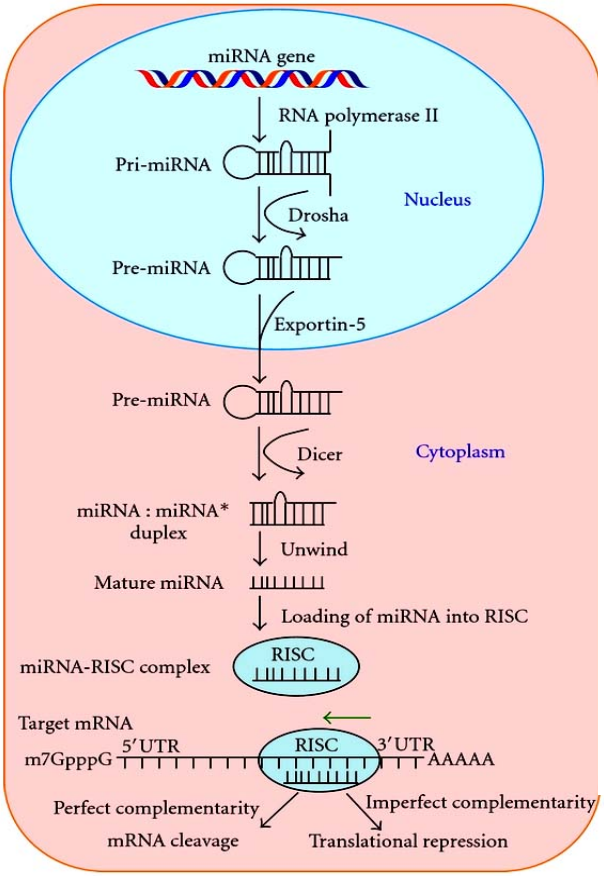


Figure 1.1: The biogenesis of microRNA in a cell. After the transcription of the miRNA gene in the nucleus, the primary transcript (pri-miRNA) is cleaved into a precursor molecule (pre-miRNA) with an imperfect stem-loop structure by Drosha. The pre-miRNA is exported from the nucleus into the cytoplasm by exportin-5. In the cytoplasm, the pre-miRNA is cleaved by Dicer into a dsRNA duplex (miRNA: miRNA*), which contains both the single-stranded mature miRNA and its complementary strand (miRNA*). The miRNA strand is then incorporated into the RNA-induced silencing complex (RISC) and targets the complementary mRNA sequences via translational repression or mRNA cleavage. Adapted from Kobayashi et al., 2012.

The pre-miRNA is exported from the nucleus into the cytoplasm by exportin-5. In the cytoplasm, RNase III enzyme Dicer interacts with dsRDB protein and cleaves

pre-miRNA to produce mature double stranded (dsRNA) ~22 nt miRNA-miRNA* duplex, which contains both the single-stranded mature miRNA and its complementary strand (miRNA*). The miRNA is then incorporated into the RISC (RNA induced silencing complex) formed by the interaction of dsRBD protein, Dicer and Argonaute proteins. The miRNA then guides the RISC to the target mRNAs by base pairing interactions and cause their translation repression or degradation or destabilization (reviewed in Bushati and Cohen, 2007) (**Fig. 1.1**).

1.5.2.2. Short interfering RNAs (siRNAs)

Also known as small interfering RNAs, is a class of long, linear, perfectly base paired double stranded RNA. These dsRNAs are processed by Dicer into 21-25 nt siRNAs that direct silencing. siRNAs were originally observed during transgene- and virus-induced silencing in plants (Mello and Conte, 2004). The siRNAs can arise from endogenous genomic loci or as products of foreign nucleic acid. The RISC loading complex (same as discussed in miRNA) binds to dsRNA, dice it into siRNA and load the siRNA into Argonaute endonucleolytic protein (Macrae *et al*., 2008). The double stranded siRNA possesses one guide strand and one anti-guide or passenger strand. The guide strand directs RISC to perfectly complementary targets, which are then, degraded causing post transcriptional gene silencing. The passenger strand is degraded as a RISC complex substrate (**Fig. 1.2**; reviewed in Carthew and Sontheimer, 2009).

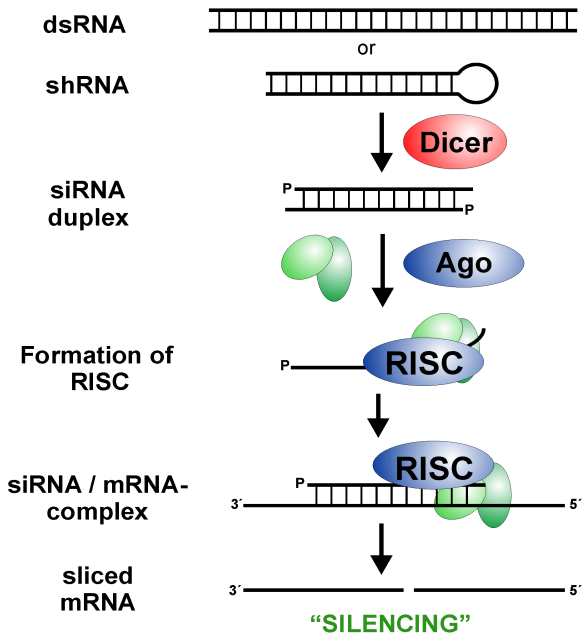


Fig. 1.2: Major steps on the biogenesis and mechanism of action of siRNA pathway: Long dsRNAs are cleaved by Dicer into siRNAs in an ATP-dependent reaction. Incorporation of siRNAs into RISC follows, and unwinding of the dsRNAs requires ATP. Once unwound, the single-stranded antisense strand guides RISC to mRNAs having a complementary sequence and results in the endonucleolytic cleavage of the target mRNAs (www.gene-quantification.de/si-rna)

As siRNA is both robust and specific, it has become an important tool for validating gene function by gene knock-down and is being developed for use in agriculture and clinical applications. For example RNAi is used to improve crops by providing resistance against parasites, and modified versions of siRNAs that are directed against disease causing genes are being developed (Engels, 2012).

1.5.2.3. piRNAs

piRNAs are also known as Piwi interacting RNAs. These are 24 to 30 nt long; asymmetric (i.e. only one strand of a sequence is represented) RNAs generated by a Dicer- independent mechanism and interact with a subset of Argonaute proteins related to Piwi. piRNAs are primarily derived from transposons and other repeated sequence elements found in clusters throughout the genome. The primary function of piRNA pathway is to repress the expression and transposition of transposable elements (Simonelig, 2011). The genetic studies in drosophila, mice and zebrafish highlight the significance of piRNAs in germline transposon regulation (Malone and Hannon, 2009; Klattenhoff and Theurkauf, 2008; Seto *et al*., 2007). **rasiRNAs** or repeat associated siRNAs, is a subclass of piRNA and are involved in regulating chromatin structure and transcriptional silencing (Aravin *et al*., 2003).

1.6. Non coding RNAs in Prokaryotes (bacteria)

Among the riboregulators of bacteria, the small RNAs and riboswitches are predominant types and modulate transcription, translation, mRNA stability, and DNA maintenance or silencing. They achieve these diverse outcomes through a variety of mechanisms, including changes in RNA confirmation, protein binding, base pairing with other RNAs, and interactions with DNA (Waters and Storz, 2009). The riboswitches are part of the mRNAs that they regulate and are predominant in prokaryotes. Recently few are reported in eukaryotes (Breaker, 2012).

The largest and the most extensively studied set of small RNA (sRNA) regulators act through base pairing with target RNAs, usually modulating their translation and stability. Others are small transcripts which bind to proteins, including global regulators, and antagonize their functions. Finally, a recently discovered group of RNA regulators, known as the CRISPR (clustered regularly interspaced short palindromic repeats) RNAs constitute the prokaryotic defence system and interfere with bacteriophage infection and plasmid conjugation by targeting the homologous foreign DNA (reviewed in Waters and StorZ, 2009). CRISPR cassette is transcribed as a continuous transcript which is processed by Cas proteins into small RNA molecules that are responsible for defence against invading viruses (Djordjevic, 2012).

1.6.1. Riboswitches

Riboswitches are structured non coding RNA domains that selectively bind metabolites and control gene expression (Coppins *et al*., 2007; Roth and Breaker, 2009). A typical riboswitch consists of two functional domains: a small molecule receptor (called the aptamer domain) and a regulatory domain (called the expression platform) (Winkler and Breaker, 2003; Garst and Batey, 2009). Both domains participate in the folding and structural rearrangement in the absence or presence of its cognate metabolite. Presently more than 20 classes of riboswitches have been reported that contain receptor domains that respond to nucleobases and nucleosides, amino acids, cofactors, amino sugars and metal ions (Breaker, 2012). The *trp, his, bgl* and few other amino acid operons of *E. coli* consist of a leader peptide which senses the concentration of required biomolecules and control the formation of a transcriptional terminator structure (reviewed in Yanofsky, 2000).

Bacterial riboswitches mediate gene regulation primarily by promoting intrinsic transcription termination or inhibiting translation initiation (Bastet *et al*., 2011; Hollands *et al*., 2012). In transcription anti-termination the ligand binds to the aptamer region and prevents the formation of the terminator stem by regulating the formation of the competing secondary structure or anti-terminator. Translationally controlling riboswitches contain aptamer region upstream of an intrinsic transcription terminator stem that is formed using Shine-Dalgarno sequences thereby occluding the ribosome binding site and/or start codon.

Few examples of riboswitches identified in *E. coli* metabolism involved in biosynthesis or transport of molecules include sensors for TPP (thiamine pyrophosphate) (Winkler *et al*., 2002a), FMN (flavomononucleotide) (Winkler *et al*., 2002b), SAM (S-adenosylmethionine) (Winkler *et al*., 2003) and Mg^{2+} ion (Cromie *et al*., 2006).

Today an ever increasing number and variety of riboswitches and their mechanisms have been uncovered in bacteria as well as eukaryotes (Breaker, 2011).

1.6.2. Small RNAs in cell physiology of bacteria

A small RNA (sRNA) is a non protein coding RNA molecule, encoded by separate transcription unit in intergenic region between known open reading frames (ORF) and carries out gene-regulatory functions. The sRNAs can act by diverse mechanisms, e.g., they can base-pair with target mRNAs and thereby repress or activate translation or influence RNA stability or they can directly bind to proteins and modulate their activities. They regulate responses to changes in environmental conditions and general stress by enabling the cell to adjust its physiology. They play a key role bacterial virulence by quickly altering the saprophytic lifestyle to the virulence mode.

1.6.3. Characteristics of bacterial sRNAs

Few general characteristics of bacterial sRNAs are as follows;

(i) Usually less than 300 nucleotide in length with few exceptions
(ii) Located in intergenic regions between known protein coding genes
(iii) Protein non coding transcripts
(iv) Rho independent transcription termination
(v) Defined secondary structure, usually conserved among related species

(vi) Majority of them regulate responses to changes in environmental conditions.

1.6.4. RNA mining: Approaches to identify novel small RNAs in bacteria

The detection of sRNA encoding genes experimentally, computationally or by mutational screens has been difficult as they are small in size and do not get translated into proteins. Most of the bacterial sRNAs reported till 2000 were detected either fortuitously due to their abundance or by discovery of a sRNA during studies on a protein or by observation of the activities associated with over expression of genomic fragments. Nevertheless, with the advent of high throughput sequencing techniques currently a large number of sRNAs in various bacteria have been predicted (Sharma and Vogel, 2009). An overview of approaches for identifying sRNAs is discussed in this section

1.6.4.1. Direct labelling and sequencing

In this method the *E. coli* total RNA was metabolically labelled with $^{32}PO_4^{3-}$ (orthophosphate), separated on one and two dimensional polyacrylamide gels and autoradiographed. The selected bands were excised and digested by ribonuclease T_1 for fingerprinting or cloned for sequencing. The sRNAs discovered by such methods included 6S RNA and Spot 42 RNA (Ikemura *et al*., 1973). In addition, the simple ethidium bromide staining of total RNA separated on polyacrylamide gel detected BS190 and BS203 sRNAs in *Bacillus subtilis* (Ando *et al*., 2002).

1.6.4.2. Genetic screens

Few sRNAs were discovered fortuitously by observations of phenotypes conferred by multicopy plasmids or by detection of RNAs during the study of particular operons. The MicF sRNA was discovered while studying the genetic regulation of the *E. coli* outer membrane proteins OmpC and OmpF (Mizuno *et al*., 1984). Likewise the study of regulatory elements involved in capsular synthesis identified the 87 nt regulatory RNA DsrA (Sledjeski *et al*., 1996). The *E. coli* sRNA CsrC and PrrB sRNA of *Pseudomonas fluorescens* were identified in functional screens for genes involved in glucan biosynthesis (Aarons *et al*., 2000; Weilbacher *et al*., 2003). Few other *E. coli* sRNAs discovered in genetic screens are RprA (Majdalani *et al*., 2001), DicF (Bouche *et al*., 1989), and UptR (Guigueno *et al*., 2001).

1.6.4.3. Biocomputational screens

The three pioneering biocomputational driven studies (Argaman *et al*., 2001; Wassarman *et al*., 2001 and Rivas *et al*., 2001) in *E. coli* extracted the sequences of IGRs (intergenic regions) from the genome and subjected them to an algorithm or a predictive scheme based on few or all of the following criteria: (i) transcription initiation signals (promoters); (ii) Rho independent transcription termination signals; (iii) sequence length of 50 bp to 400 bp and its conservation among closely related species; (iv) candidate gene orientation and (v) conservation of potential RNA secondary structure. The predicted sRNAs were examined by northern analysis and their both ends were mapped by 5' and 3' RACE (rapid amplification of cDNA ends). Another study confined the search to transcriptional signals and restricted the length of predicted sRNAs to 45 nt - 370 nt for sRNAs in *E. coli* (Chen *et al*., 2002). Other algorithms developed to predict sRNAs include sRNAPredict, RNAz, ISI (intergenic sequence inspector) (Livny *et al*., 2006; Washietl *et al*., 2005; Pichon and Felden, 2005). Further, use of multilayered computational searches and high throughput technologies predicted hundreds of sRNAs in various bacteria (Livny *et al*., 2008; Weinberg *et al*., 2007).

1.6.4.4. Co-purification with Proteins or Target RNAs

Several sRNAs associate with proteins for their biological activity or stability or act on and modify the activity of their target proteins. Many such sRNAs were identified upon co-immunoprecipitation with protein complexes. The sRNAs, CsrB of *E. coli* and RsmZ of *Pseudomonas fluorescens* were identified by co-purification with their target proteins, CsrA and RsmA, respectively (Liu *et al*., 1997; Heeb *et al*., 2002). Most sRNAs in bacteria which require Hfq protein for intracellular stability and target mRNA pairing were identified by co-immunoprecipitation with anti-Hfq antibodies and subsequent hybridization to tiling arrays. The *E. coli* sRNAs discovered during protein studies include OxyS, GcvB, Crp Tic RNAs (Vogel and Wagner, 2007).

1.6.4.5. Microarray detection

DNA microarray technique enables the simultaneous genome wide monitoring of the gene expression. It became a very powerful tool to detect sRNAs in various micro organisms by use of probes specific to IGRs where most sRNAs are located. The high-density microarray was developed for *E. coli* which had probes for all ORFs,

tRNAs and rRNAs as well as for both the clockwise and counterclockwise strands of each IGRs. The use of these microarrays and total RNA extracts or RNAs isolated by co-immunoprecipitation with the RNA binding protein, Hfq identified several sRNAs (Tjaden *et al*., 2002; Wassarman *et al*., 2001; Zhang *et al*., 2003; Hu *et al*., 2006). However nowadays tiled microarray with full genome coverage have transcended the conventional microarray for sRNA searches.

The tiling microarray is a high resolution genome array where the probe sequences are simply derived sequentially, moving along the genome, without regard to sequence features but skipping the repeats. The first tiling array in *E. coli* covered IGRs > 40 bp (with one probe every six base pairs) in addition to the strand specific probes for all annotated regions of genome (Selinger *et al*., 2000). Thereafter the tiling arrays have led to the global discovery of sRNAs in a large number of microorganisms.

1.6.4.6. RNomics and next generation sequencing (RNA-Seq)

In RNomics, also called as shotgun cloning, cDNA libraries were made of RNA extracted under different growth conditions and fractionated on denaturing polyacrylamide gels. The cDNA library was screened for potential sRNA candidates (Vogel *et al*., 2003). Next, RNA-Seq also called as "Whole Transcriptome Shotgun Sequencing was used to detect sRNAs. It uses high-throughput sequencing technologies to sequence cDNA in order to get information about sample's RNA content. In general, a population of RNA (total or fractionated, such as poly(A)+) is converted to a library of cDNA fragments with adaptors attached to one or both ends. Each molecule, with or without amplification, is then sequenced in a high-throughput manner to obtain short sequences from one end (single-end sequencing) or both ends (pair-end sequencing). The reads are typically 30–400 bp, depending on the DNA-sequencing technology used (Wang *et al*., 2009). More than ~60 small RNAs were identified in *Helicobacter pylori* by primary transcriptome profiling using RNA-seq (Sharma *et al*.,2010) and new sRNAs in *Pseudomonas syringae* (Filiatrault *et al*., 2010) and *Pseudomonas aeruginosa* (Gomez-Lozano *et al*., 2012) were reported.

1.7. Small RNA in various pathogenic bacteria

After the prediction of hundreds of regulatory sRNAs in *E.coli*, similar screens were conducted in various other bacteria. As the sRNAs are reported to play a key role in

pathogenicity by regulatory mechanisms controlling host tissue invasion, nutrient acquisition and evasion of the host immune defence systems (Sharma and Heidrich, 2012), searches were mostly concentrated on pathogenic bacteria. A large number of sRNAs were predicted in *Listeria monocytogenes*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Bacillus subtilis, Mycobacterium tuberculosis* and many others. The sRNAs in few bacterial species are discussed below.

1.7.1. sRNAs in *Salmonella typhimurium*

Salmonella typhimurium is a gram negative, pathogenic bacterium closely related to *E. coli* K12. It invades and replicates in eukaryotic cell and causes disease in a variety of mammalian and non mammalian hosts. At least 70 small RNAs have been identified in Salmonella and more than half of them are homologs of *E. coli* sRNAs (Papenfort *et al*., 2006; 2008). A recent study used the combination of three RNA sequencing techniques and identified 60 new sRNAs expressed at early stationary phase in *S.typhimurium* (Kröger *et al*., 2012). As 25% of genome of *S.typhimurium* is laterally acquired and constitutes pathogenicity islands, a search was conducted to identify small RNAs in these genetic islands. Total 19 island-encoded novel small RNAs were identified using predictive algorithm (Padalon-Brauch *et al*., 2008). A few examples of characterized *S.typhimurium* sRNAs include, GcvB, RybB, CyaR, InvR, CsrB/C, MicA, RyhB, SgrS, IsrJ etc. For example, CyaR sRNA (~86 nt) regulates *ompX* mRNA encoding a small abundant porin that is highly over produced in *hfq* mutants (Sittka *et al*., 2008). One other sRNA, InvR encoded by *invR* gene located in *Salmonella* invasion gene island SPI-1 regulates the expression of *ompD* which encodes an outer membrane protein (Pfeiffer *et al*., 2007). The *Salmonella* homologue of *E. coli* sRNA GcvB, regulates the expression of multiple ABC transporters of amino acids and peptides (Sharma *et al*., 2007). The several studies conducted in recent past indicate that many sRNAs in *Salmonella* play an important role in regulation of the expression of genes required for bacterial adaptation to environmental changes and stress conditions and hence control virulence of the organism. Moreover improved understanding of sRNA biology will help to elucidate precise role of sRNAs during the infection process and *Salmonella* pathogenicity (Hébrard *et al*., 2012).

1.7.2. sRNAs in *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive pathogen and most common cause of hospital acquired infection and a wide range of human diseases. *S. aureus* express numerous virulence factors and stress response molecules which are regulated at multiple levels during the course of infection. RNA III (486 nt), the first regulatory sRNA discovered in *Staphylococcus* acts on mRNAs by antisense mechanism and regulates the synthesis of virulence related genes and plays a key role in quorum sensing-dependent regulatory circuit (Ji *et al*., 1995; Huntzinger, 2005; Boisset *et al*., 2007). Comparative genomics and bioinformatics approaches predicted 12 sRNAs, five from core genome i.e. 4.5S RNA, tmRNA, RNase P, RNAIII and 6S RNA, seven from the pathogenicity islands which were called as Spr (small pathogenicity island rRNAs) i.e. SprA, SprB, SprC, SprD, SprE, SprF and SprG (Pichon and Felden, 2005) and 11 novel, *hfq* independent non coding RNAs (RsaA-K) expressed at late exponential phase of growth (Geissman *et al*., 2009). 30 sRNAs, including 14 that were newly confirmed were identified by investigating the RNome of *S. aureus* using experimental approach (Bohn *et al*., 2010). The *S. aureus* SprD sRNA negatively regulates the expression of immune-evasion molecule Sbi by antisense mechanism, occluding the Shine-Dalgarno (SD) sequence and initiation codon (Chabelskaya *et al*., 2010). Another sRNA RsaE was found to control the ABC transporter operon by targeting the SD sequences of two of its cistrons, *opp3A* and *oppB* mRNA, both encoding components of ABC transporter operon (Geissman *et al*., 2009; Bohn *et al*., 2010). The RsaE-dependent downregulation of numerous metabolic enzymes involved in the citrate cycle and folate-dependent one-carbon metabolism was suggested by combination of biocomputational and transcriptional analyses (Bohn *et al*., 2010).

1.7.3. sRNAs in *Pseudomonas aeruginosa*

Pseudomonas is a gram negative opportunistic pathogen. After identifying several sRNAs in *E. coli* using bioinformatics tools, many researchers expanded their searches for sRNAs in several other bacteria. In 2006, Livny *et al*., developed a bioinformatics tool, sRNAPredict and used it to identify candidate sRNA-encoding genes in the IGRs of *P. aeruginosa*. About 17 genes encoding sRNAs were confirmed after northern blot validation. Two independent searches in 2008 involving RNomics, structure based bioinformatics tools, biocomputational approaches and

experimental validations identified many novel sRNAs (Sonnleitner *et al*., 2008; González *et al*., 2008). In 2012, the RNA sequencing (RNA-seq) involving three different sequencing libraries identified more than 500 novel intergenic sRNAs in *P. aeruginosa* (Gómez-Lazona *et al*., 2012).

Out of these, P28 likely corresponded to *P. aeruginosa* RnpB, which in *E. coli* is a component of RNase P, the enzyme involved in the processing of 4.5S RNA and tRNA precursor molecules (Livny *et al*., 2006). However not all *Pseudomonas* sRNAs are homologous to *E. coli* sRNAs. For example, PrrF RNAs bear no resemblance to the equivalent RyhB sRNA of *E.coli*, although both are repressed by Fur and act on similar targets (Wilderman *et al*., 2004). Two other sRNAs, RsmY and RsmZ of strain PAO1 are involved in quorum sensing and bacterial pathogenesis (Heurlier *et al*., 2004; Sonnleitner *et al*., 2006). The induction of another sRNA PhrS led to increased levels of expression of genes required for synthesis of the virulence factor pyocyanin (PYO). The genes for PYO are under the regulation of common transcriptional regulator PqsR, the key quorum-sensing regulator. It was found by genetic studies that *phrS* base pairs and activates a short upstream open reading frame to which the *pqsR* gene is translationally coupled (Sonnleitner *et al*., 2011; Sharma and Storz, 2011).

1.7.4. sRNAs in *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *Vibrio cholerae* and several other micro organisms

Two independent searches involving *in silico* tools, *hfq* dependent coimmuoprecipitation followed by chemical RNA sequencing and experimental validation identified nearly 15 small RNAs in *L .monocytogenes* (Christiansen *et al*., 2006; Mandin *et al*., 2007). A few examples of *L. monocytogenes* sRNAs include LhrA, LhrB, LhrC and nine sRNAs from RilA-RilI. Few of these sRNAs were absent in non pathogenic *L. innocua* strain suggesting their probable role in virulence. The sRNA LhrA inhibits translation of *lmo0850* mRNA at post transcriptional level. The effects of LhrA when studied on global wide gene expression indicated it to be a global regulator and nearly 300 genes were positively or negatively affected (Nielsen *et al*., 2011). The recent searches discovered 103 sRNAs expressed in *L. monocytogenes* growing under a wide range of conditions from broth culture to blood and intestinal lumen (Toledo-Arana *et al*., 2009). One other search involving deep

sequencing of cDNAs obtained from fractionated RNAs identified more than 150 putative regulatory RNA (Mraheil *et al*., 2010). The functional characterization of most of these sRNAs is currently under investigation.

In *M. tuberculosis*, sRNAPredict2 tool predicted 56 sRNAs and screening the cDNA libraries identified nine putative sRNAs, four *cis*-encoded (ASdes, ASpks, AS1726 and AS890) and five *trans-encoded* sRNAs B11, B55, C8, F6, G2, (Livny *et al*., 2006; Arnvig and Young, 2009). Both these studies could not find any *E. coli* Hfq RNA chaperone homologues in *M. tuberculosis* suggesting the probability of an alternative pathway. Most of these sRNAs showed complementarity to multiple genes in *M. tuberculosis* and overexpression of two sRNAs, B11 and G2 were found to be lethal suggesting their potential regulatory characteristics as exhibited by their counterparts in other bacteria (Arnvig and Young, 2009). The combination of RNA-seq technique and comparative genomics recently predicted 1948 sRNAs in *M. tuberculosis* (Pellin *et al*., 2012).

In *V. cholerae*, numerous sRNAs have been predicted using sRNAPredict tool and few of them were confirmed by northern analysis (Liu *et al*., 2009). One of the sRNAs, VrrA of *V. cholerae* positively regulates the release of outer membrane vesicles through down regulation of outer membrane protein OmpA (Song *et al*., 2008). Four redundant regulatory *V. cholerae* sRNAs called quorum regulatory RNAs (Qrr1-4) regulate the expression of *hapR* mRNA and numerous other genes required for virulence. Three other sRNAs CsrB, CsrC and CsrD act redundantly to abolish the activity of global regulatory protein CsrA. All these seven sRNAs regulates the expression of quorum-sensing cascade in *V. cholerae* (Lenz *et al*., 2004; Lenz *et al*., 2005).

Recent advances in bioinformatics and the availability of the complete genome sequence have led to the identification of numerous small RNAs in various other microorganisms such as *Helicobacter pylori*, *Yersinia spp* and *Streptococcus pyogenes* (Sharma and Heidrich, 2012).

1.8. sRNAs in *Escherichia coli*

The various approaches to predict small RNAs in bacteria led to identification of hundreds of small RNAs in *E. coli* and experimental validation of nearly 90 small RNAs in *E. coli* (Raghavan *et al*., 2012).

The discovery of *E. coli* sRNA was marked by the discovery of antisense RNAs, RNAI and CopA that controlled copy number by base pairing in ColEI and R1 plasmids respectively. The separation of ^{32}P-orthophosphate labelled RNA on one and two dimensional gel electrophoresis and RNase T_1 fingerprinting identified 6S, 4.5S, Spot 42 and 10S sRNAs (Hindley, 1967; Griffin, 1971; Ikemura and Dahlberg, 1973). The 6S RNA associates with RNA polymerase holoenzyme and represses expression of sigma 70 dependent promoters during stationary phase while 4.5S RNA is a part of signal recognition particle. The Spot 42 sRNA was characterized as a negative regulator of translation of *galK* (galactokinase) of *gal* operon (Møller *et al*., 2002). The 10S RNA was found to comprise two species 10Sa and 10Sb now known as tmRNA and RNase P-M1 RNA respectively.

Another sRNA MicF inhibited translation of the mRNA encoding OmpF, the major outer membrane porin (Mizuno *et al*., 1984; Andersen *et al*; 1989). Later DicF (Bouche and Bouche, 1989), DsrA (Sledjeski and Gottesman, 1995), CsrB (Liu *et al*., 1997) and OxyS (Altuvia *et al*., 1997) sRNAs were discovered. The functional characterization of these sRNAs indicated that DsrA activates and OxyS negatively regulates the translation of *rpoS* mRNA encoding stationary phase *E. coli* sigma factor. The CsrB sRNA was found to interact with translational regulatory protein CsrA (Liu *et al*., 1997).

Today with the high throughput technologies and next generation sequencing, more than 90 small RNAs in *E. coli* have been identified and validated (Waters and Storz, 2009; Raghavan *et al*., 2011).

1.8.1. sRNAs that modulate protein activity

Few *E. coli* sRNAs (CsrB, 6S RNA and GlmY) function by binding to a protein to modulate its activity. These regulatory sRNA mimic the structures of other nucleic acids which help them to interact with target proteins (**Fig. 1.3**).

The CsrB and CsrC sRNAs of *E. coli* contain conserved repeating sequence motifs required to bind to their target protein CsrA, an RNA-binding protein that regulates carbon usage and bacterial motility upon entry into stationary phase and other nutrient poor conditions (reviewed in Babitzke and Romeo, 2007).

The expression of *glmS* transcript (encodes for glucosamine-6-phosphate synthase) is translationally repressed by its 5'-UTR structure and is regulated by two small RNAs, GlmZ and GlmY. GlmZ sRNA interacts with the *glmS* transcript and unmasks the ribosome binding site (RBS) and promotes translation. Additional modulation is provided by YhbJ, a protein involved in GlmZ turnover and thus negatively regulates GlmS expression by decreasing the amount of GlmZ available to activate *glmS*. GlmY, a second sRNA has significant sequence and structural homology to GlmZ such that when it is expressed, GlmY acts as a decoy, binding YhbJ. Thus, GlmY prevents GlmZ degradation increasing GlmZ concentration and allowing more *glmS*-GlmZ adduct formation, with a net result of enhancing GlmS expression (reviewed in Gőrke and Vogel, 2008). One more sRNA, 6S, interacts and modulates the activity of a protein which is discussed in section 1.8.2. 6S RNA is only known sRNA that acts at the transcription level (Trotochaud and Wassarman, 2005)..

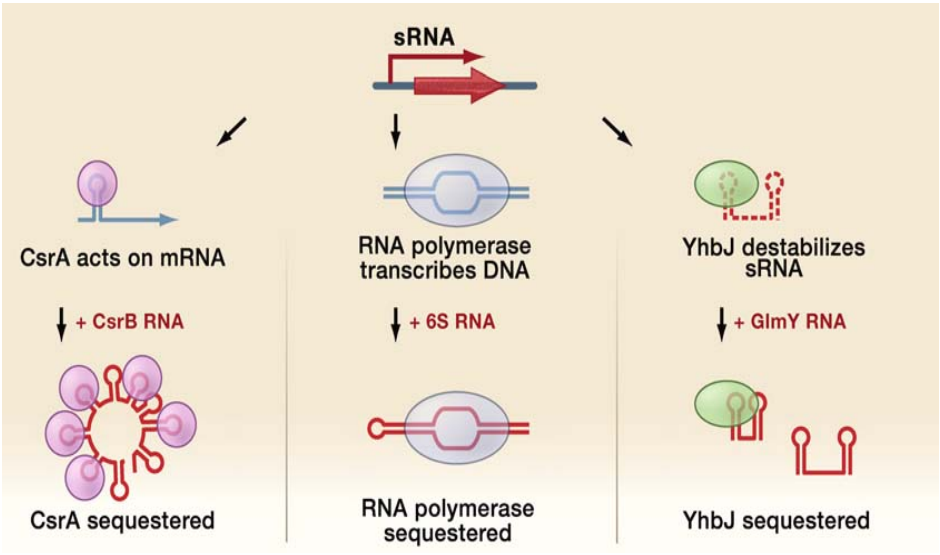


Fig. 1.3: sRNAs modulating protein activity: Some *trans*-encoded sRNAs interact with proteins, including transcription factors, and inhibit their activity (Adapted from Waters and Storz, 2009).

1.8.2. sRNAs that modulate transcription

All of the sRNAs characterized till date function post transcriptionally. The only exception is 6S sRNA which has a direct effect on the transcriptional apparatus. The *E. coli* 6S sRNA, which accumulates in stationary phase, was the first sRNA to be sequenced about 30 years ago. The 6S sRNA mimics an open promoter and binds to the σ^{70}-bound, housekeeping form of RNA polymerase but not to the σ^{S}-bound, stationary phase form of RNA polymerase. The interaction between 6S and σ^{70} holoenzyme reduces transcription from certain σ^{70}promoters and increases transcription from σ^{S}-regulated promoters, thus help in altering the promoter recognition during stationary phase (Trotochaud and Wassarman, 2005).

1.8.3. sRNA modulating translation

In contrast to the few known protein binding sRNAs, most characterized sRNAs act as antisense regulators and regulate gene expression by base pairing with mRNAs, affecting stability or translation of the message. The base pairing sRNAs fall into two broad classes: (i) *cis* encoded: sRNAs which are encoded in *cis* on the DNA strand opposite the target RNA and (ii) *trans* encoded: sRNAs encoded far from their targets.

1.8.3.1. *cis*-encoded base pairing sRNAs

The *cis*-encoded sRNAs also called as natural antisense sRNAs (asRNAs) share extended regions of complete complementarity with their target and are often 75 nucleotides or more. The majority of *cis* -encoded antisense RNAs have been found in plasmids, phages and transposons. The plasmid encoded antisense RNAs are expressed constitutively and regulate fundamental processes as replication initiation, conjugation efficiency, suicide or transpositions

A recent transcriptome analysis confirmed the widespread antisense transcription in *E. coli* by identifying about 1000 different antisense sRNA (asRNAs) (Dornenburg *et al*., 2010). Bacterial asRNAs are diverse and can be classified based on their location as 5'-overlapping (divergent, head to head), 3'-overlapping (convergent, tail to tail), or internally located asRNAs (reviewed by Brantl, 2007; Georg and Hess, 2011). The chromosomally encoded antisense RNAs have been found to be expressed only under certain conditions, for example GadY in stationary phase (Opdyke *et al*., 2004) or IsrR under iron stress (Duhring *et al*., 2006) or at high levels of toxic proteins. The GadY sRNA is encoded by a gene located in the middle of the *gad* gene cluster with

gadX and *gadW* on either side that are part of a complex regulatory circuit controlling *E. coli* response to acid stress. The base pairing of GadY small RNA with the intergenic region of the *gadX-gadW* mRNA results in directed processing by RNase III and their subsequent accumulation at higher levels than unprocessed mRNA (Opdyke *et al*., 2011).

Several *cis* acting sRNAs in *E. coli* (SibA-E, SymR) have been characterized as antitoxins belonging to type I toxin-antitoxin pair (Kawano *et al*., 2007) and have similar modes of regulation as observed for well characterized plasmid sRNAs that are involved in maintenance and replication. These sRNAs are encoded in *cis* and repress the expression of ORFs encoding toxic proteins. For example, the *E. coli* SymR sRNA is transcribed in *cis* to the 5' end of *symE*, a SOS induced protein and tightly controls its synthesis. The SymR antisense RNA base pairs with SymE and influences mRNA stability and represses translation (Kawano *et al*., 2007). In *E.coli*, there are also two other sRNAs, OhsC and IstR, that are encoded directly adjacent to genes encoding potentially toxic proteins (Fozo *et al*., 2008) (**Fig 1.4**).

Besides *E.coli*, several other bacteria possess *cis*-encoded antisense RNA. For example, in *V.cholerae*, MtlS sRNA is transcribed in *cis* and antisense orientation relative to the 5' UTR of *mtlA* encoding the mannitol-specific transporter of the PTS. MtlS shares 70 nt perfect complementarity with *mtlA* transcript and represses the translation and/or the stability by direct base pairing (Liu *et al*., 2009). Another example of antitoxin/toxin system leading to antisense mediated mRNA degradation is of the RatA/TxpA pair of *B.subtilis* (Silvaggi *et al*., 2005). In *Synechocystis*, antisense small RNA IsrR negatively impacts the level of oppositely encoded *isiA* mRNA. The synthesis of IsrR is repressed by iron stress which allows accumulation and translation of *isiA* mRNA (Duhring *et al*., 2006).

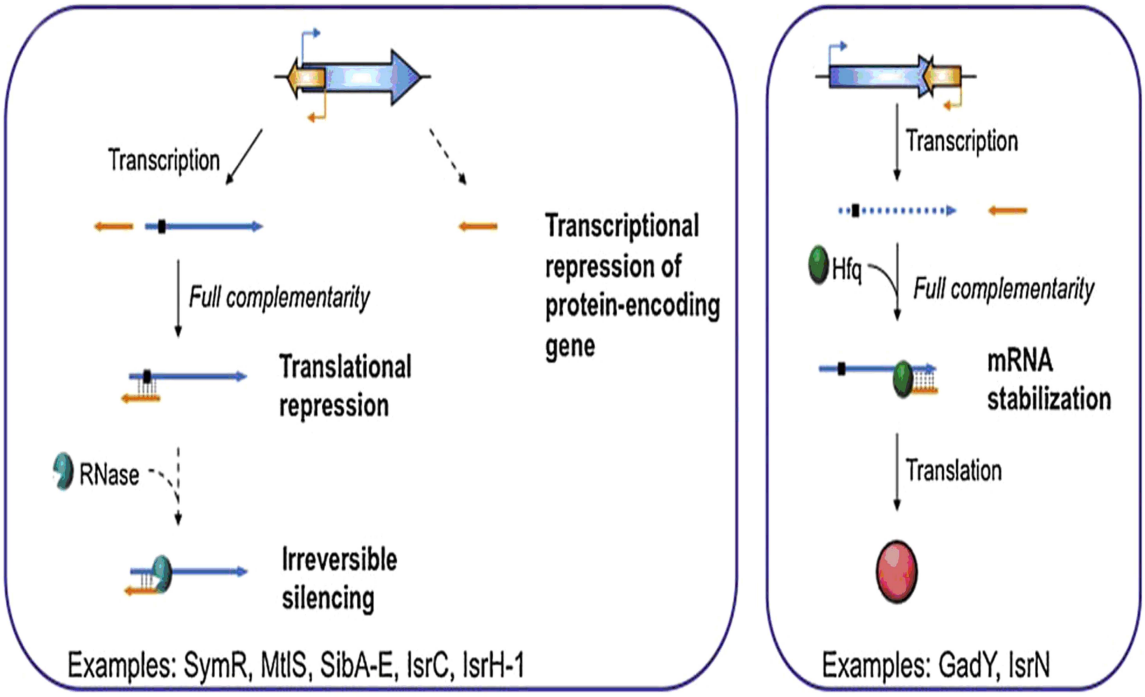


Fig. 1.4: *cis*-encoded antisense sRNAs: These can either (a) repress or (b) activate protein expression. (Adapted from Liu and Camilli, 2010). The colored arrows represent RNA transcripts; black boxes indicate Shine-Dalgarno (SD) sequences. Dashed color arrows represent unstable transcripts. Dashed black arrows represent hypothetical mechanistics steps of sRNA-mediated regulatory pathways.

1.8.3.2. *trans*-encoded base pairing sRNAs

The *trans*-encoded sRNAs are encoded distant from their targets and share only limited complementarity with them. These sRNAs are functionally analogous in many ways to eukaryotic miRNAs and are known to regulate the translation and/or stability of target mRNAs, either positively or negatively (reviewed in Aiba, 2007; Gottesman, 2005).

Although majority of *trans*-encoded antisense RNAs repress the translation, a few sRNAs activate the expression of their target mRNAs by base pairing and disrupting an inhibitory secondary structure which sequesters the ribosome binding site (reviewed in Waters and StorZ, 2009). The best known *E. coli* sRNAs DsrA (Sledjeski and Gottesman, 1995) and RprA (Majdalani *et al*., 1998) stimulate the translation of target transcripts by base pairing with the upstream leader region of *rpoS* mRNA that relieves the inhibition by releasing the Shine Dalgarno site

(Majdalani *et al*., 1998; Majdalani *et al*., 2002). Another sRNA RyhB activates the translation of *shiA* mRNA encoding a permease of shikmate, a compound involved in siderophore synthesis (Prévost *et al*., 2007). The RNAIII sRNA of *S. aureus* also positively regulates the translation of α-toxin while negatively regulating other genes (Morfeldt *et al*., 1995).

The majority of the regulation by the known *trans*-encoded sRNAs is negative (reviewed by Waters and Storz, 2009). The sRNAs base pair with their target mRNAs followed by translational inhibition, mRNA degradation or both. The bacterial sRNAs primarily bind to the 5' UTR of their target mRNAs and most often occlude the ribosome binding site and such sRNA-mRNA duplex are then subjected to degradation by RNaseE. For example, OxyS sRNA represses translation of RpoS as well as FlhA transcripts by base pairing near the ribosome binding site (RBS) on the transcript and blocks ribosome binding (Altuvia *et al*., 1998). The *E. coli* MicA sRNA inhibits OmpA (outer membrane protein) synthesis through base pairing with ribosome binding sequence (Udekwu *et al*., 2005). MicA also pairs with *phoPQ* mRNA in the translation initiation region of *phoP* mRNA and thus repress the *E. coli* pleiotropic PhoPQ two component system (Coornaert *et al*., 2010). The RprA sRNA which is a positive regulator of RpoS translation however negatively regulates translation of two mRNAs, *csgD* and *ydaM* encoding a stationary phase-induced biofilm regulator and a diguanylate cyclase respectively (Mika *et al*., 2012).

Some sRNAs such as GcvB and RyhB, inhibit translation through base pairing far upstream of the AUG of their target genes. The GcvB sRNA regulates expression of several genes- *sstT*, *oppA*, *dppA* and *cycA*- involved in transport of amino acids and peptide (Urbanowski *et al*., 2000; Pulvermacher *et al*., 2008; Pulvermacher *et al*., 2009; Sharma *et al*., 2011). A recent report suggested that GcvB can also bind to the 5' UTR of the *csgD* mRNA, the transcriptional regulator of curli synthesis and act as a repressor (Jørgensen *et al*., 2012). The RhyB sRNA negatively regulates Fur expression, the main repressor of iron uptake genes and more than 18 operons encoding 56 genes (Massé *et al*., 2005). Additionally RyhB downregulates the translation of a small upstream open reading frame (uof) that is translationally coupled with Fur and hence provides the first example of indirect translation regulation by *trans*-encoded sRNA (Večerek *et al*., 2007) (**Fig 1.5**).

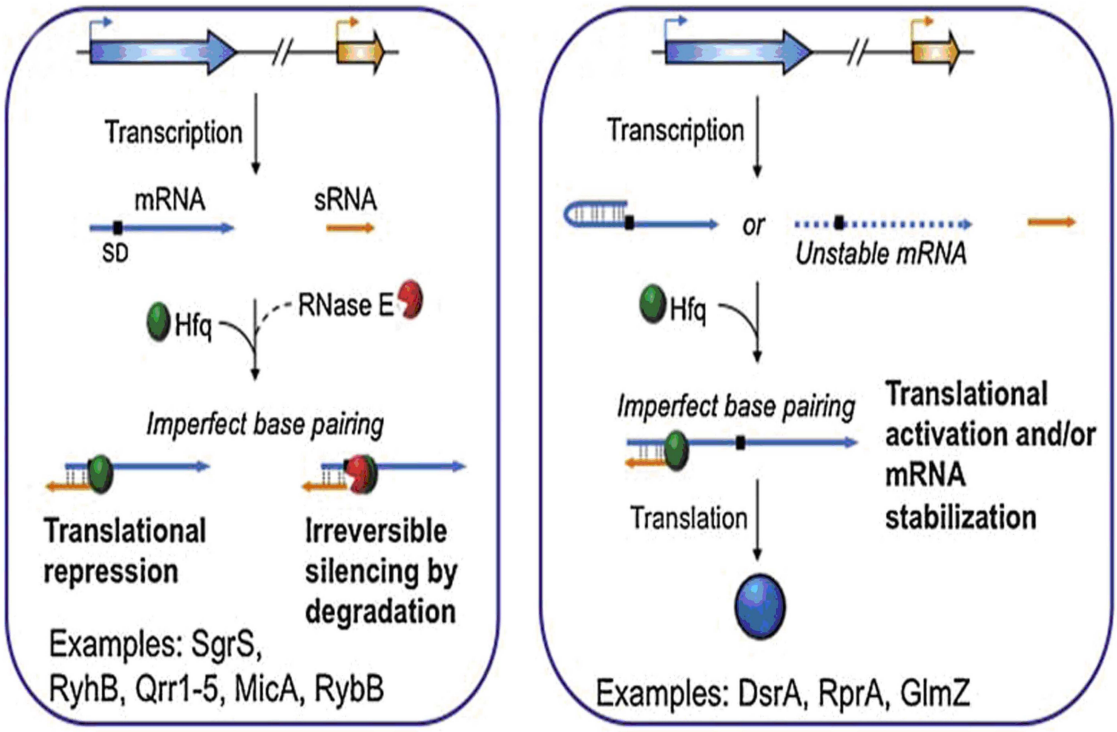


Fig. 1.5: *trans*-encoded sRNAs: These base pair imperfectly with mRNA targets and either (a) repress or (b) activate translation (Adapted from Liu and Camilli, 2010). The colored arrows represent RNA transcripts; black boxes indicate Shine-Dalgarno (SD) sequences. Dashed color arrows represent unstable transcripts. Dashed black arrows represent hypothetical mechanistics steps of sRNA-mediated regulatory pathways.

Most of the *trans*-encoded sRNAs are synthesized under specific growth conditions. For example, in *E.coli*, the RyhB is induced by low iron, OxyS is activated by oxidative stress, MicA and RyhB are induced by stress at outer membrane. Besides stress conditions, concentrations of specific molecules also activates expression of small RNAs. Elevated glycine induces GcvB, changes in glucose concentration activate Spot 42 and CyaR and elevated glucose-phosphate levels activate SgrS small RNA (reviewed by Waters and StorZ, 2009).

As in most cases the complementarity between the *trans*-encoded sRNA and its target transcript is only partial or less, a majority of them bind and require RNA chaperone molecule Hfq. The role of Hfq and other proteins related to small RNAs are discussed in section 1.9.1.

1.9. Proteins required for sRNA function

1.9.1. Hfq protein

All the characterized *trans*-encoded sRNA mediated regulations in Gram-negative bacteria require Hfq protein, presumably to facilitate RNA-RNA interactions due to limited complementarity between the sRNA and target mRNA (reviewed by Vogel and Luisi, 2011). The Hfq protein (**H**ost **f**actor involved in phage **Q**β- replicase function) is a pleiotropic regulator of several genes in *E. coli* (Tsui *et al*., 1994) and was first identified as a host factor, along with ribosomal protein S1, required for replication of an *E. coli* bacteriophage **Q**β (Blumenthal and Carmichael, 1979). Hfq, the Sm-like (RNA binding) hexameric protein was found to possess two binding sites; the proximal site, which binds sRNA and mRNA; and the distal site, which binds AU-rich region, with a preference for binding next to a structured (stem loop) region (reviewed by Brennan and Link, 2007). The properties of *hfq* mutants led to recognition that Hfq was necessary for translation of RpoS, the stress sigma factor of *E. coli* and was shown to be important for overcoming an inhibitory hairpin upstream of *rpoS* (Brown and Elliot, 1997).

The Hfq facilitates sRNA-mRNA interactions by increasing annealing rates (Fender *et al*., 2010; Hopkins *et al*., 2011; Hwang *et al*., 2011) by stabilizing cognate sRNA-mRNA duplexes (Soper *et al*., 2010) or by promoting structural remodelling of one of the RNA partners (Maki *et al*., 2010). An acceleration in binding rate due to Hfq was clearly demonstrated in the interaction between the sRNA SgrS and its target *ptsG* mRNA (Kawamoto *et al*., 2006; Maki *et al*., 2010) and the rate of binding for in vitro synthesized MicA-*ompA* mRNA (Rasmussen *et al*., 2005) (**Fig. 1.6**).

Many sRNAs are stabilized by Hfq in vivo and its absence caused RNase E mediated degradation of least few sRNAs (Folichon *et al*., 2003). For example Hfq binding protects DsrA and RyhB sRNAs from cleavage by RNase E.

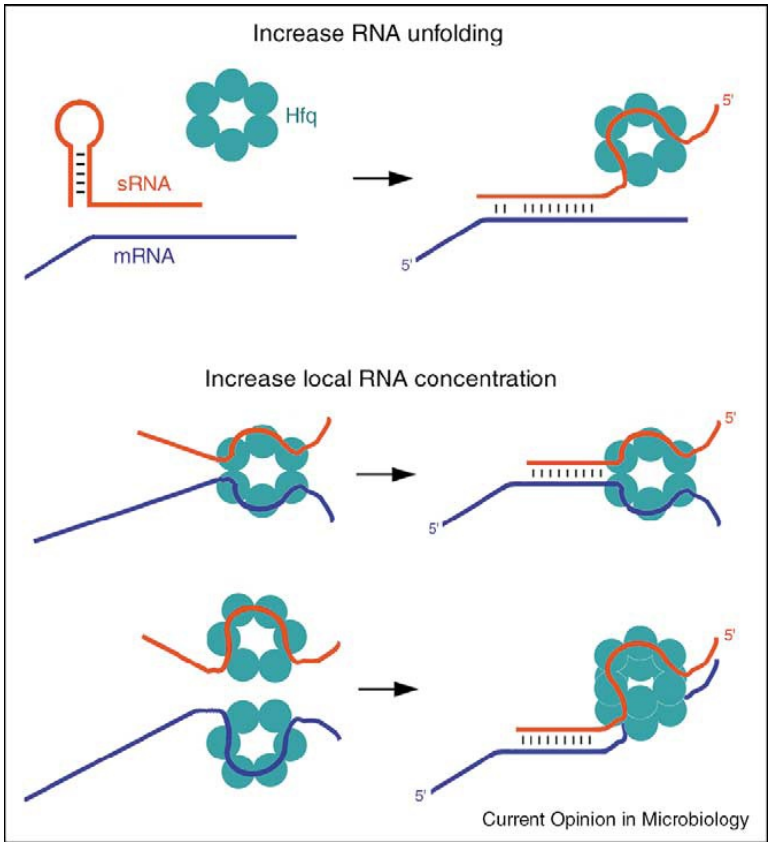


Fig. 1.6: Hfq mediated sRNA-mRNA base pairing: Mechanisms by which Hfq might facilitate sRNA-mRNA base pairing (Adapted from Storz *et al*., 2004). Hfq (aqua ring) may promote RNA unfolding or may increase the local concentrations of the sRNA (red) and its mRNA (blue).

1.9.2. Ribonucleases

The major outcome of sRNA-mRNA interaction is degradation of the complex brought about by Ribonucleases. Both, RNase E and RNA III endonucleases are known to cleave base pairing sRNAs and their targets (Viegas *et al*., 2007).

RNase E apart from being an essential enzyme involved in processing many RNAs, most significantly tRNA, RNaseP and tmRNA, was also found to play a critical role in the function of Hfq-binding sRNAs. Interestingly, like Hfq, RNase E also recognizes AU- rich sequences and adjacent stem loop structures (Moll *et al*., 2003). However once Hfq promotes the annealing of sRNA with the target RNA, the complex is degraded in RNase E dependent manner (Massé *et al*., 2003).

The RNase E preferentially cleaves within single stranded regions and the site of cleavage could be adjacent to the sRNA-target mRNA duplex as observed for MicC-directed cleavage of the *ompD* mRNA (Pfeiffer *et al*., 2009) or at a significant

distance from the region of duplex formation as found for RyhB and *sodB* (Prévost *et al*., 2011).

RNase E has been implicated in the degradation of several sRNA-mRNA duplexes. For example, the regulation of *E. coli ptsG* mRNA (encoding sugar phosphate transporter) by small RNA SgrS involves both Hfq and RNase, where Hfq binds to RNase E and SgrS associates with RNase E through Hfq to form a specific ribonucleoprotein complex and degrades *ptsG* mRNA (Morita *et al*., 2006).

RNase III has also been shown to be involved in few sRNA mediated interaction. One such sRNA is IstR which base pairs with a short region in the *tisAB* mRNA and this antisense interaction entails RNase III dependent cleavage, thereby inactivating mRNA for translation (Vogel *et al*., 2004). The *E. coli* MicA appears to be degraded by RNase E when not base-paired and by RNase III when paired with target mRNAs (Viegas *et al*., 2011).

1.10. Elucidation of sRNAs targets

Once a large number of *E. coli* sRNAs were identified the quest to identify their targets began. The true target for an sRNA was aptly defined as an mRNA or protein which the sRNA physically interacts and whose function, stability or translation it affects: that is, an mRNA that is translationally activated, inhibited or degraded as a consequence of being bound by an antisense RNA is confirmed as a true (primary) target of the sRNA (Sharma and Vogel, 2009).

The sRNA targets could be predicted computationally or experimentally. For computationally based predictions, algorithms have been developed that predict plausible interaction sites for query sRNAs and thereby find candidate targets. A large number of target prediction programs are available which include TargetRNA, RNAhybrid, RNAplex, RNAup, IntaRNA, and sTarPicker (Ying *et al*., 2011). However computational predictions of targets are only complementary to the experimental validation of these targets.

The experimental approaches to identify mRNA targets of sRNAs include physiological studies upon over expression or disruption of the sRNA, point mutation strategies, reporter gene assays, microarray profiling and proteomics-based approaches.

Table 1.1: List of E. coli sRNAs and their known targets and function

sRNA	Target name: Target Description; Reference
DicF	***ftsZ*****:** Essential cell division protein; Septal ring GTPase required for cell division and growth; initiation of septation; (Tetart *et al*., 1992; Tetart and Bouche, 1992)
DsrA	***rpoS*** **:** (Lease *et al*., 1998; Majdalani *et al*., 1998; Majdalani *et al*., 2002; Sledjeski *et al*., 1996)
DsrA	***Hns*** **:** DNA-binding global regulator H-NS; (Lease *et al*., 1998; Sledjeski and Gottesman, 1995; Urban and Vogel, 2007)
GadY	***gadX*****:** Transcriptional activator for *gadA* and gadBC, AraC family; (Opdyke *et al*., 2004)
GcvB	***oppA*****:** Oligopeptide ABC transporter substrate-binding; (Tjaden *et al*., 2006; Urbanowski *et al*., 2000)
GcvB	***dppA*****:** Dipeptide/heme transport, periplasmic binding protein; (Tjaden *et al*., 2006; Urban and Vogel, 2007; Urbanowski *et al*., 2000)
GcvB	***csgD:*** *S*tationary phase-induced biofilm regulator (Jørgensen *et al*., 2012).
Istr1	***tisAB*** **:** The locus codes for two genes : TisA (unconserved 37 amino acid putative peptide) and TisB (29 amino acid peptide widely conserved) ; (Vogel *et al*., 2004)
MicA	***ompA*****:** Outer membrane protein A; Discovered to influence biofilm formation; (Rasmussen *et al*., 2005; Udekwu *et al*., 2005; Urban and Vogel, 2007)
MicC	***ompC*****:** (Chen *et al*., 2004; Urban and Vogel, 2007)
MicF	***ompF*****:** (Aiba *et al*., 1987; Andersen and Delihas, 1990; Mizuno *et al*., 1984; Ramani *et al*., 1994; Urban and Vogel, 2007)
OmrA	***fecA*****:** Ferric citrate outer membrane porin (Guillier and Gottesman, 2006)
OmrA	***cirA*****:** Outer membrane receptor for iron-regulated colicin I receptor; porin; (Guillier and Gottesman, 2006)
OmrA	***ompT*****:** Outer membrane protease VII; (Guillier and Gottesman, 2006)
OmrA*	***fepA*** **:** Outer membrane receptor for ferric enterobactin (enterochelin) and colicins B and D; (Guillier and Gottesman, 2006)
OxyS	***fhlA*****:** Formate hydrogen lyase system activator, global regulator; (Altuvia *et al*., 1998; Argaman and Altuvia, 2000)
OxyS	***rpoS*****:** RNA polymerase subunit, stress and stationary phase sigma S; (Altuvia *et*

	al., 1997; Zhang *et al*., 1997; Zhang *et al*., 1998)
OxyS	***yobF* :** Predicted protein ; (Tjaden *et al*., 2006)
OxyS	***wrbA* :** NAD(P)H:quinone oxidoreductase; (Tjaden *et al*., 2006)
OxyS	***ybaY*:** Novel verified lipoprotein, function unknown; (Tjaden *et al*., 2006)
RprA	***rpoS* :** (Majdalani *et al*., 2001; Majdalani *et al*., 2002)
RprA	***csgD:*** Stationary phase-induced biofilm regulator (Mika *et al*., 2012)
RprA	***ydaM*: D**iguanylate cyclase respectively (Mika *et al*., 2012).
RybB	***ompC*:** (Johansen *et al*., 2006)
RybB	***ompW*:** Outer membrane protein W; Component of Colicin S4 transport system; (Johansen *et al*., 2006)
RybB	***rpoE*:** RNA polymerase subunit, extracytoplasmic stress sigma E; role in high temperature and oxidative stress response; (Thompson *et al*., 2007)
RydC	***yejABEF*:** Putative ABC transporter; (Antal *et al*., 2005)
RyhB	***Fur*:** Fe DNA-binding transcriptional dual regulator of siderophore biosynthesis and transport ; (Vecerek *et al*., 2007)
RyhB	***sodB*:** Superoxide dismutase, Fe; response to oxidative stress; (Masse and Gottesman, 2002; Urban and Vogel, 2007)
RyhB	***sdhC*:** Component of membrane-bound subcomplex of succinate dehydrogenase; (Masse and Gottesman, 2002)
RyhB	***sdhD*:** Component of membrane-bound subcomplex of succinate dehydrogenase; (Masse and Gottesman, 2002)
RyhB*	18 operons encoding 56 genes (Massé *et al*., 2005)
SgrS	***ptsG*:** Glucose phophotransferase enzyme IIBC(Glc); glucose permease; (Kawamoto *et al*., 2006; Kawamoto *et al*., 2005; Kimata *et al*., 2001; Morita *et al*., 2005; Urban and Vogel, 2007; Vanderpool and Gottesman, 2004)
Spot42	***galK*:** Galactokinase; (Moller *et al*., 2002; Urban and Vogel, 2007)
SymR	***symE*:** Hypothetical conserved protein; (Kawano *et al*., 2007)

1.11. Present study

Bacterial sRNAs regulate the expression of a variety of genes by base pairing with

target mRNAs and leading to repression or activation of translation or mRNA degradation. To date more than 1000 sRNAs have been identified in *E. coli* but the direct targets of approximately only 50 sRNAs have been validated (Ying *et al*., 2011). The number of characterized sRNA targets has increased slowly in comparison to steady increase in the number of sRNAs identified. The precise targets of most sRNAs remain elusive.

The sRNA targets could be predicted computationally or experimentally. In some cases, targets were found by simple BlastN searches, such as, the *ompC* or *tisAB* mRNAs which are regulated by MicC or IstR-1 sRNAs respectively. However computational predictions of targets are only complementary to the experimental validation.

The functional characterization of few *E. coli* sRNAs with unknown function would help in understanding the complex regulatory pathways of how bacteria uses these small RNAs to fine tune their physiology and adapt to the rapidly changing environment. Moreover, it would also contribute to the existing knowledge of the mechanism involved in the macromolecular interactions.

Objective of the work

i) To search for putative targets of few selected uncharacterized *E. coli* sRNAs using bioinformatics tools.

ii) To carry out molecular manipulations such as overexpression, disruption, specific deletion, substitution etc of selected sRNAs.

iii) To analyze the altered expression of target genes in the presence of manipulated cognate sRNAs.

In the present work 20 sRNAs were selected for bioinformatics and computational analysis for target prediction. The analysis showed hypothetical proteins as targets for many sRNAs while known protein genes as targets for a few. The sRNAs, RyjA and IsrC were selected for their functional characterization. Both these sRNAs are *cis*-encoded. The RyjA overlaps the transcription termination sequences at the 3' end of SoxR while IsrC overlaps the 5' regulatory region of Antigen 43. The selected sRNAs were overexpressed or disrupted and the associated phenotypic changes or altered levels of their target mRNAs were estimated. The 3’ region of RyjA involved

in base pairing interactions and regulation of expression of SoxR was also analyzed.

Identification of potential targets of several uncharacterized *E. coli* sRNAs:

At the beginning of this work around 100 *E. coli* sRNAs with known and unknown function were reported. Out of them about 20 sRNAs with unknown function were selected and subjected to BLAST search.

BLAST analysis. A summary of BLAST analysis of 20 selected sRNAs is given below.

Table 1.2

sRNA(bp)	Similarity Match(a.a.)	Alignment Display	Identities
IS102(204)	**Antigen 43 (1091)**	**ncRNA 43--------2** **Target 5--------18**	**92%**
IS061(158)	No significant hit		
rybA(89)	Hypothetical protein c0902(42)	ncRNA 44--------88 Target 1--------15	100%
rydB(68)	No significant hit		
ryeA(249)	Hypothetical proteinnc2248(249)	ncRNA 2--------118 Target 12-------50	94%
ryeD(143)	Hypothetical protein ECs 3934(130)	ncRNA 6--------113 Target 91-------128	50%
ryeE(85)	No significant hit		
rybB(79)	**Putative regulator TetR/Acr Family (209)**	**ncRNA 79-------37** **Target 192-------206**	**92%**
ryg(76)	Hypothetical protein c3433 (155)	ncRNA 76-------2	100%

		Target 37-------111	
sraG(174)	Polyribonucleotide nucleotidyltransferase(740)	ncRNA 55-------41 Target 60--------102	92%
ryeC(149)	Hypothetical protein ECs 3934(130)	ncRNA 6-------119 Target 91------128	57%
ryeB(121)	Hypothetical protein c2248(150)	ncRNA 2-------88 Target 22------50	96%
ssrA(363)	Hypothetical protein(130)	ncRNA 294-------259 Target 20------54	65%
Tke(149)	Thiosulfate sulfertrasferase(278)	ncRNA 81-------57 Target 2---------25	66%
IS092(160)	Hypothetical protein c2317(48)	ncRNA 129-------1 Target 1---------43	95%
sraD(76)	No significant hit		
ryjA(140)	**Redox sensitive transcriptional activator soxR(154)**	**18nt termination sequences which overlap that of soxR**	
sraB(169)	Hypothetical protein c1357(42)	ncRNA 22-------81 Target 1---------20	95%
ryhA(108)	Sigma cross reacting protein 27A(220)	ncRNA 51------37 Target 8------- 50	100%

Table 1.2 BLAST analysis of the *E.coli* sRNAs.

Material and Methods

2.1. Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 2.1 and Table 2.2 respectively. The sequence and restriction maps of the vectors are given in appendix.

Table 2.1

Bacterial strains	Genotype and relevant characteristics	Reference-Source
Escherichia coli DH5α	(F⁻ Φ80 d*lacZ*ΔM 15 Δ(*lacZYA-argF*) U169 *endA*1 *recA*1, *hsdR*17 (*rk*⁻ , *mk*⁺)) *deoR thi*-1 *supE*44 *λ*⁻ *gyrA*96 *relA*1	Lab stock
E. coli KL 16	*Hfr*, Wild type strain	Lab stock
E. coli BW1042	GC4468 λΦ ((*soxR'-'lacZ*) *bla*⁺)	Gaddu and Weiss, 2000.
E. coli BW1157	GC4468 λΦ ((*soxS'-'lacZ*) *bla*⁺)	Gaddu and Weiss, 2000
E. coli MC1061	F⁻ *araD139* Δ *(ara-leu) 7696 galE15 galK16* Δ*(lac)X74 rpsL* (Str^r) *hsdR2(*$r_k^-m^+$*) mcrA mcrB1*	Lab stock
E. coli DY330	W3110 Δ*lacU169 gal490 λcI857* Δ(*cro-bioA*)	Yu *et al*. (2000)
E. coli RyjADM	DY330 Δ*ryjA::kan* (Kan^r)	This work
Uropathogenic E. coli (UPEC)	Isolated from Health centre, M.S. University of Baroda, Vadodara (Gujarat, India)	This work

Table 2.1. List of bacterial strains used in this study: The name, description and reference /source for each strain are given.

Table 2.2

Plasmid/Phage	Features	Size (bp)	Reference
pNEB206A	*E. coli* vector, *lac* promoter ; Amp^r	*2722 / 2706	New England Biolabs,USA
pBluescript	Cloning vector for *E. coli*; Amp^r	3000	Stratgene,USA

KS+			
pBluescript SK-	Cloning vector for *E. coli*; Amp^r	3000	Stratgene, USA
Plasmid/Phage	**Features**	**Size (bp)**	**Reference**
pBR322	Cloning vector for *E. coli*; Amp^r	4360	Sutcliffe, (1979)
pBBR1MCS-2	Broad-Host-Range vector; originally derived from pBBR1 plasmid of *Bordetella bronchispetica*; Km^r	5144	Kovach *et al*., (1995)
pWB53	pLDR10::Φ(*soxS'-'lacZ*) Amp^r ,Cam^r		Gaddu and Weiss, 2000
pMNC10	pNEB206A encoding *ryjA* sRNA, Amp^r	2876	This work
pMNC10T	pMNC10 with tetracycline marker, Tet^r	4815	This work
pMNC10KS	pBSKS$^+$ encoding *RyjA* sRNA, Amp^r	3170	This work
pNEB206AT	pNEB206A with tetracycline resistant marker, Tet^r	4615	This work
pRyjA2	pMNC10T with 10-45 nt deleted from *RyjA*, Amp^r, Tet^r	4780	This work
pRyjA3	pMNC10T with 110-140 nt deleted from RyjA, Amp^r , Tet^r	4785	This work
pRyjAS3	pMNC10T with modified 110-140 nt of *RyjA,* Amp^r , Tet^r	4815	This work
pRyjAKan	pMNC10, Kanamycin marker between cloned *RyjA*, Amp^r, Km^r	4480	This work
pAntiIsrC	pBluescript KS+ encoding antisense of *isrC* sRNA , Amp^r	3240	This work
pIsrC	pBluescript SK- encoding *isrC* sRNA, Amp^r	3240	This work

Table 2.2. List of plasmids/phage used in this study: NEB=New England Biolabs;

* The circular pNEB206A vector is 2722 bp long. The linear plasmid is 2706 bp. Amp= Ampicillin; Tet= Tetracycline; Km= Kanamycim; r = resistant.

2.2. Media, chemicals, enzymes, biochemicals and kits

Media and general chemicals were obtained from HiMedia Laboratories, India; Qualigens, India or Sisco Research Laboratories, India. Analytical grade chemicals were procured from Merck, India and Sigma Chemicals Pvt. Ltd., USA.

All enzymes and molecular biology grade biochemicals were from Applied Biosystems, USA; Bangalore Genei Pvt. Ltd, India (now Merck Lifesciences, India); MBI Fermentas, Germany; New England Biolabs (NEB), USA; Promega, USA; Roche Diagnostics, Germany and Sigma Chemicals Pvt. Ltd., USA.

2.3. Media and Culture conditions

2.3.1. Media

Luria broth (LB) and Nutrient broth (NB) were obtained as a readymade medium from Hi-Media Laboratories. LB at 2% (w/v) and NB at 1.3 % (w/v) was used according to the manufacturer's instructions. 2 % (w/v) agar was added to prepare respective solid agar medium. All these media were autoclaved at 15 lbs for 15 minutes.

2.3.2. Culture conditions

All bacterial strains were cultured and maintained on Luria Agar (LA) at 37 ºC unless mentioned specifically. Cultures were grown in broth by shaking at 200 rpm. Measurements of growth as optical density at 600 were carried out on Elico SL171 mini spectrophotometer. Bacterial strains were maintained as slants or stabs at 4°C. For long term preservation, the cultures were maintained as 20% glycerol stocks and stored at -20°C. Antibiotics, IPTG, X-gal and Methyl viologen (MV) were used at concentrations indicated in Table 2.3.

Table 2.3

Biochemical	Stock Concentration (mg/ml)	Final concentration (µg/ml)
Ampicillin	100	100
Tetracycline	10	10
Kanamycin	40	40
IPTG	20	20
Methyl Viologen	50 mM	100 µM
ONPG	4	400
Pyrogallol	25	65
X-Gal	20	40

Table 2.3. Concentration of antibiotics, IPTG, X-gal and Methyl Viologen (paraquat).

2.3.3. IPTG and Paraquat induction

The overnight grown cultures were diluted to an OD_{600} of 0.02 in LB with appropriate antibiotics and were grown 37ºC with constant shaking (200 rpm). The cultures were induced with IPTG at OD_{600} of 0.4 and Methyl Viologen at OD_{600} of ~ 0.6 and were grown till an OD_{600} of ~1. 1 ml or 0.5 ml of culture was withdrawn for RNA isolation.

2.4. Molecular biology tools and techniques

2.4.1. Isolation of plasmid and genomic DNA

Plasmid DNA was isolated from overnight grown cultures by standard alkaline lysis/ boiling lysis method (Sambrook and Russell, 2001). For few experiments plasmid isolation was done by the plasmid purification kit from Roche Diagnostics or from Sigma Chemicals Pvt. Ltd. Genomic DNA from *E. coli* was isolated by standard procedure (Sambrook and Russell, 2001).

2.4.2. PCR

2.4.2.1. PCR conditions

The PCR reaction set up was based on the guidelines given in Sambrook and Russell 2001. The assay system and temperature profiles used are described in Table 2.4.

Oligonucleotide sequences used in present study were custom synthesized from Sigma Aldrich Chemicals Pvt. Ltd., MWG Biotech Pvt. Ltd. India and are listed in Table 2.5.

Table 2.4.

Assay system used		Temperature profile	
Sterile DDW	13 µl	Initial denaturation	94°C for 2 min.
dNTP (10mM with 2.5mM each)	2.0 µl	Denaturation	94°C for 45 sec.
Forward Primer 10pmoles	1.0 µl	Annealing	Varies from 55-62°C for 30sec.*
Reverse Primer 10pmoles	1.0 µl		
Template DNA (100ng/µl)	0.5 µl	Extension	72°C for 45sec-2.5min#
Taq PCR buffer (10X)	2.0 µl	Final extension	72°C- 10 min
X-Taq or Taq DNA Polymerase	0.5 µl	For plasmid	(30 cycles)

(3 units/µl)#			
Total System	20 µl	For genomic DNA	(40 cycles)

Table 2.4. PCR conditions used in the present study. PCR amplifications were performed in Eppendorf mastercycler personal or Eppendorf mastercycler gradient thermal cycler. *Exact primer annealing temperature and primer extension time varied with primers designed with respect to different templates and has been specified in the text as and when applicable.

2.4.2.2. List of Primers

Table 2.5.

Primer	Primer sequence	Restriction sites
A.	**Primers for amplification of *ryjA* and *soxR*:**	
RyjAFP	GGAGACAUATCAACACCAACCGGAACC	
RyjARP	GGGAAAGUCGCTTGCTGGTGGAAGATGAAC	
SoxR1FP	CGCGGATCCCTGCCTCTTTTCAGTGTTCAG	BamHI
SoxR1RP	CGCAAGCTTCCGGAAAACAAACTAAAGCGC	HindIII
B.	**Primers for amplification of kanamycin resistance gene:**	
KanFP	CCCGAAAAGTGCCACCTG	
KanRP	GAGAGGCGGTTTGCGTATT	
C.	**Primers for *ryjA* stem 2 and stem 3 deletion**	
RyjA2FP	TGCACTGCAGTGCAATCAACACCAACCGGAACCTCCACCACG TGCTCGAATGAGGTGTGTTGAAAGACCGG	PstI
RyjA2RP	CGAGCTCGAAACTAAAGCGCCACAAGGGCGCTTTAGTTTGTT TTCCGGTCTTTCAACACACCTCATTCG	SacI
RyjA3FP	TGCACTGCAGTGCAATCAACACCAACCGGAACCTCCACCACG TGCTCGAATGAGGTGTGTTGACGTCGGGGGAAACCC	PstI
Primers	**Primer Sequence**	**Restriction sites**
RyjA3RP	CGAGCTCGTGTTTTCCGGTCTTTGTCTTTCTCTCTATCCCGC TGGTACACAGGAGGGTTTCCCCCGACGTCAACAC	SacI
D. Primers for *ryjA* 3' end substitution:		
RyjAS3FP	TGCA*CTGCAG*TGCAATCAACACCAACCGGAACCTCCACCAC GTGCTCGAATGAGGTGTGTTGACGTCGGGGGAAACCCTCCT GTGTACCAGCGGGATA	PstI

RyjAS3RP	CGAGCTCGAAGATGAACAAAACAATACGCGGCGAAGCCGCG TATTGTTTTGTTTTCCGGTCTTTGTCTTTCTCTCTATCCCG CTGGTACACAGGA	SacI
E. Primers for Real time PCR		
SoxRFP	AACAGCTTTCGTCCCAATGG	
SoxRRP	AGGCAGCCACAACCAATACAT	
SodAFP	GCCTGGATGTGTGGGAACAT	
SodARP	GCTGCTTCGTCCCAGTTCAC	
EndoFP	CAAGGTGTGACAGCGGTGAT	
EndoFP	TATCTTCCACGCCGTCGATAA	
16SFP	ATACCGCATAACGTCGCAAGA	
16SRP	GTGAGCCGTTACCCCACCTA	
F. Primers for *isrC* amplification:		
IsrCFP	CGCGGATCCGTCCGTGCAATAGCTCAAT	BamHI
IsrCRP	CGCAAGCTTCAGATGTCGTTTCATCAGC	HindIII
G. Primers for *flu* gene amplification:		
Agn43FP	CCGTTAATCAGAAGGGCAGA	
Agn43RP	GCGGGTATTAGTGGCTGTGT	
16S rRNAFP	ATACCGCATAACGTCGCAAGA	
16S rRNARP	GTGAGCCGTTACCCCACCTA	

Table 2.5. List of primers used in present study. The DNA sequences recognized by restriction enzymes are shown in boldface print. FP = Forward primer, RP = Reverse primer.

2.4.3. Restriction enzyme digestion

The DNA was mixed with 5-10 U of restriction endonuclease (RE) per microgram of DNA in 1 X buffer supplied by the manufacturer in a final reaction volume of 20 – 100 µl. The reaction mixtures were incubated for 2- 16 h at the appropriate temperature. In case of double digestion, a compatible buffer for the two REs was essentially used when available.

2.4.4. Agarose gel electrophoresis

The DNA samples were mixed with appropriate volume of 6X loading buffer (0.25% bromophenol blue, and 40% sucrose in water) and subjected to electrophoresis through 0.8 - 2 % agarose gel in 0.5X Tris-borate-EDTA (TBE) buffer at 4 V/cm for 1-3 hrs. The gels were then stained for 30- 45 minutes in 0.5X TBE containing 5 µg/ml ethidium bromide (EtBr). The DNA bands were visualized by fluorescence under UV light using a UV transilluminator and subsequently photographed using gel docking apparatus.

2.4.5. Elution of DNA from gels and purification

The DNA fragments of desired sizes were cut out from 0.8% low melting point agarose gel without exposing the DNA to ethidium bromide or UV light, by running a parallel sample for EtBr staining and visualization. The agarose piece was digested with β-agarase (NEB) and recovered by precipitation. In some cases, the DNA fragment of interest was purified using GenElute Agarose spin columns from Sigma Chemicals Pvt. Ltd. The purified DNA was checked by subjecting about 60 ng of the DNA solution to gel electrophoresis and visualizing the DNA band of interest.

2.4.6. Processing with T4-DNA polymerase

1 µg of DNA was heat shocked at 65˚C for 5 min and then cooled on ice. To the reaction vial 4 µl of 5X reaction buffer, 1 µl of dNTP mix (2 mM each) and 0.2 µl of T4 DNA polymerase (1 unit) (MBI Fermentas) were added. The volume was made upto 20 µl with nuclease free sterile water, mixed thoroughly and incubated at 11˚C for 20 min. The enzyme was then inactivated by heating at 75˚C for 20 min.

2.4.7. Ligation

The ligation reaction was usually carried out with Quick ligase (NEB), T4 DNA ligase (Bangalore Genei or MBI Fermentas) according to the manufacturers' instructions with vector to insert molar ratio of 1:3 for cohesive end and 1:8 for blunt end ligations, with a total of 50-100 ng of vector DNA in each ligation system. The ligation mixtures were incubated at the temperatures and times recommended by the manufacturer.

2.4.8. Transformation of plasmid DNA in *E. coli*

Plasmid DNA was transformed into *E. coli* DH5α using the $CaCl_2$ method (Sambrook and Russell, 2001). The transformants were selected by plating on selection medium

containing appropriate antibiotic and/or by blue white selection using IPTG and X-Gal.

2.4.9. Cloning by using USER (uracil-specific excision reagent) friendly cloning kit

pNEB206A was obtained as a linearized vector 2,706 bp in length (with bp 438-453 excised from the circular form) flanked by two non-complementary 8-base 3' overhangs at the intended cloning site. Amplification with deoxyuridine anchored primers and subsequent treatment with USER enzyme results in PCR product with 3' overhangs complementary to those in pNEB206A. These products are directionally cloned into pNEB206A without the use of restriction enzymes or DNA ligase, forming recombinant circular molecules (Appendix).

2.4.10. Electroporation

2.4.10.1. Preparation of electrocompetent cells

The overnight culture was diluted to an OD_{600} of 0.02 in 50 ml LB medium and was grown at 32°C with shaking. When the culture reached an OD_{600} of 0.4-0.6, 10 ml of culture was induced and centrifuged for 8 min at 5,500 X g at 4°C. The pellet was resuspended into 1 ml of ice cold sterile water and was spun for 20 sec at 4°C at maximum speed in a microfuge. The cells were washed thrice with and finally resuspended in 100 µl of ice cold sterile water and were immediately used for electroporation.

2.4.10.2. Electroporation

Purified linear donor DNA (50-100 ng) was mixed with induced electrocompetent cells (described in section 2.4.10.1) and pipetted into a precooled electroporation cuvette. Electroporation was performed by using a Bio-Rad Gene Pulser set at 1.8 kV, 25 µF with pulse controller of 200 ohms. The electroporated cells were immediately diluted with 1 ml LB medium, incubated at 32°C for 1-2 hr and then spread on LA medium supplemented with appropriate antibiotic.

2.4.11. DNA sequencing

Purified plasmid or PCR amplicon were custom sequenced from Merck Lifesciences, India. The complete double-stranded nucleotide sequences of the inserts of sRNA expressing clones and *RyjA* deletion mutants were determined by single pass analysis.

2.5. Northern blot

2.5.1. Total RNA isolation

Total RNA was isolated from bacterial cultures at desired optical density using kits or hot phenol method (Sambrook and Russell, 2001). Proper precautions were taken to avoid RNAses contamination.

2.5.2. RNA gels and transfer

2.5.2.1. Urea polyacrylamide gel

7 M Urea/ TBE/ 8% polyacrylamide gel composition for 100 ml	
Acrylamide: bis acrylamide solution (45%)	17.8 ml
10X TBE	10 ml
H_2O	36.9 ml
Urea	42 g

All the reagents were combined and the solution was heated at 55ºC water bath for 3 min to help the dissolution of urea. The solution was removed from water bath and was allowed to cool at room temperature for 15 min with intermittent swirling. 3.3. ml of freshly prepared 1.6% APS was added and 50 µl of TEMED was added to the gel solution, gently mixed and polymerised in the pre set glass plates. The gel was pre run for 15 min at 10 volts/cm in 0.5X TBE before loading the RNA sample.

About 10-15 µg of total RNA in approximately 15 µl volume was mixed with equal volume of formamide gel loading buffer (4.9 ml of formamide, 1 ml of 0.5 M EDTA and 0.0013 g of bromophenol blue) and denatured at 70ºC for 5 min and immediately quenched on ice. The denatured RNA was resolved on 7 M urea/ TBE/ 8% polyacrylamide gel until the bromophenol dye had migrated to the bottom of the gel. The RNA was then transferred to positively charged Hybond nylon membrane (Amersham Pharmacia, England) by electroblotting in 1X TBE at 300 mA for 4 hours

at 16ºC. The RNA transferred to nylon membrane was cross linked by UV (254 nm wavelength) in a UV transilluminator for appropriate time and processed further.

2.5.2.2. Formaldehyde agarose gel

Formaldehyde agarose gel	
Agarose	1.5 gm
MOPS Buffer (10X)	10 ml
Formaldehyde	18 ml
R/O water	Make the final volume upto 100 ml

2.2.M formaldehyde / 1.5% agarose gel was prepared in MOPS buffer and pre run in 1X MOPS buffer for 5 min at 5 volts/cm before loading the RNA samples. The RNA along with the denaturants was incubated at 55ºC for 15 min and cooled.

RNA denaturation	
RNA (upto 20 µg)	25 µl
10X MOPS buffer	4 µl
Formaldehyde	8 µl
Formamide	4 µl
H_2O	9 µl

5 µl of 10X formaldehyde gel loading buffer [(50% (v/v) glycerol (diluted in DEPC-treated H_2O), 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 10 mM EDTA (pH 8.0)] was added to the reaction vial and loaded on the pre run gel. The gel was run at 4-5 volts/cm till the bromophenol dye had migrated to the bottom. The RNA was then transferred to the nylon membrane in 20X SSC for 4 hrs.

2.5.3. Probe labelling

The DNA fragment was amplified, purified, labelled and quantified using DIG High Prime Labelling and Detection Kit from Roche Diagnostics. The kit uses digoxigenin (DIG), a steroid hapten, to label DNA fragments for hybridization and subsequent color detection by enzyme immunoassay. DIG-labelled DNA probes are generated by the random primed labelling technique. About 2 µg of DNA template in 16 µl sterile water was then denatured by heating in a boiling water bath for 10 min and then quickly chilled on ice. 4µl of 5X DIG-High prime mix was added to the DNA template and incubated overnight at 37°C. The labelled double stranded DNA (dsDNA) probe was quantified as per manufacture's instruction and stored at - 20°C.

2.5.4. Hybridization and Detection

Approximately 100 ng probe/100cm^2 of membrane was hybridized overnight at 55ºC with gentle shaking. The membrane was washed 2 × 5 min in ample 2X SSC, 0.1% SDS at 15-25°C under constant agitation followed by 2 × 15 min in 0.5X SSC, 0.1% SDS (pre warmed to wash temperature) at 65-68°C under constant agitation. The membrane was later incubated in blocking solution for 30 min at room temperature with constant agitation. Blot was then incubated for 30 min in 1:20,000 diluted anti-Digoxigenin antibody conjugated to alkaline phosphatase and detected by chemiluminescent substrate.

2.6. Real time PCR

2.6.1. cDNA synthesis

About 2-4 µg of purified bacterial total RNA was treated with 5 U of DNaseI (MBI Fermentas) at 37°C for 30 minutes. DNaseI was inactivated by adding 1 µl of 0.25 M EDTA and incubating at 65°C for 10 minutes. RNA was quantified using a Nanodrop ND-1000 spectrophotometer and was also run on 0.8% agarose gel to check the quality and integrity of the sample. The cDNA was prepared using Genei^{TM} RT-PCR kit, Merck Lifesciences, India. 2-3 µg of DNA free RNA in 9 µl sterile DEPC treated water and 1 µl of random hexamer was incubated at 65°C for 10 min and then at room temperature for 2 min. The vial was spun briefly and the below reagents were added for single strand cDNA synthesis in the following order:

1 µl RNase inhibitor

1 µl 0.1 M DTT
4 µl Reverse transcriptase buffer (5X)
2 µl 30 mM dNTP mix
0.5 µl AMV Reverse transcriptase
1 µl sterile water

The solution was mixed well and incubated at 42°C for 1 hour. The vial was then incubated at 95°C for 2 min to denature RNA-cDNA hybrids, spun briefly and quickly placed on ice.

2.6.2. Quantitative Real time PCR

Each cDNA sample was diluted 1:5 in water and 1µl was used as the template for each 15 µl reaction mixture. The primers for *soxR*, *sodA*, *nfo* and 16S rRNA genes were designed by using Primer Express software (Applied Biosystems, USA) to generate approximately 100 bp PCR products with annealing temperature of 61°C. All the primer sequences are listed in Table 2.5. Quantitative PCR was carried out in two independent experiments using 2X Power SYBR Green master mix and PCR was monitored using Step One Real time PCR systems (Applied Biosystems, USA). 16S rRNA served as the endogenous control. Each qRT-PCR run was done in triplicate and for each reaction, the calculated threshold cycle (Ct) was normalized to the Ct of the 16S gene amplified from the corresponding sample and the fold change was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.7. Superoxide dismutase (SOD) induction/Assay

The SOD activity was measured by the percentage inhibition of auto oxidation of Pyrogallol according to the method of Marklund and Marklund (1974). Overnight grown cultures were diluted to an OD_{600} of 0.02 in 100 ml LB with appropriate antibiotics and were grown 37ºC with constant shaking. The cells were pelleted down at OD_{600} of 1, washed twice and resuspended in 10 ml 1X PBS. The resuspended cells were sonicated for 5 seconds five times in an ice bath. This was followed by centrifugation at 7,000 rpm for 10 minutes at 10ºC to remove the cell debris. The supernatant was collected in a fresh tube and stored at 4°C till further analysis. 1 mL of assay mixture containing phosphate buffer (0.2 M, pH 8), with and without cell lysate was put in a cuvette and adjusted to zero absorbance at 420 nm. The Pyrogallol main stock solution was 25mg/ml in 0.5 N HCl and working stock was diluted to 1:20

dilution. After addition of 50 µL of diluted Pyrogallol, the increase in absorbance was measured at 0.36 min intervals for 3 min on Helios spectrophotometer.

2.8. Beta Galactosidase assay

Beta Galactosidase was assayed in whole cells as per Miller (1972). Overnight grown cultures were diluted to an OD_{600} of 0.02 in LB with appropriate antibiotics and induced with IPTG. The cultures were incubated at room temperature with constant shaking to reach an OD_{600} of 0.2 and induced by Methyl Viologen and the beta galactosidase assay was performed. 0.1 ml culture was withdrawn periodically and added to the assay system containing 0.9 ml of Z-buffer (0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.01 M KCl and 0.001 M $MgSO_4$), 50 µL of chloroform and 50 µL of 3 mg/ml chloramphenicol, vortexed for 30 sec and incubated for 5 minutes at room temperature. 0.1 ml of 4 mg/ml ONPG was added to the assay system and incubated at room temperature (~30°C) till a light yellow color developed. The reaction was stopped by adding 0.5 ml of 1 M Na_2CO_3. The samples were centrifuged for 5 min and the absorbance was measured at 420 nm in Helios spectrophotometer. Specific β-galactosidase activity, expressed as nmol 2-nitrophenol/min/mg protein was calculated with the following formula.

$$\text{Specific } \beta\text{- galactosidase activity} = \frac{\text{A420 x V1}}{\text{V2 x T x 0.00486 x mg protein}}$$

Where, A420 is the OD at 420 nm, V1= final volume in ml of the assay (1.5 ml), V2 = volume in ml of the culture used in the assay (0.3 ml), and T = reaction time (in min). The molar absorption coefficient of 2-nitrophenol is 4860 M^{-1} cm^{-1} at pH 10.

2.9. Estimation of protein

Protein was estimated by the method of Lowry et al., 1951 using bovine serum albumin (BSA) as the standard.

2.10. Autoaggregation assay

The cultures were induced with IPTG and grown for 12 hrs, 24 hrs, 36 hrs and 48 hrs in flasks with shaking at 37ºC. 5 ml of culture grown for 12 hrs, 24 hrs, 36 hrs and 48 hrs was transferred to a test tube and adjusted to approximately 1 OD_{600}. At the beginning of each experiment, the cultures were vigorously shaken for 10 seconds and were kept standstill. Two 100-µl samples approximately 1 cm from the top were

carefully withdrawn from the tubes without disturbance after 12 hrs, 24 hrs, 36 hrs and 48 hrs, and transferred to two new tubes, each containing 1 ml of 0.9% NaCl. The OD_{600} was then measured (Hasman et al., 1999).

2.11. ELISA assay

2.11.1. Release and purification of Ag43 protein

The cell mass from 15 ml of culture was grown to desire optical density, harvested and gently washed in cold 1X phosphate-buffered saline. The cell suspension was placed in a 60°C water bath for 10 min in order to release the Ag43 α fragment. The cells were immediately removed by centrifugation, and the proteins in the supernatant were precipitated with acetone (75%, vol/vol). The pellet was dried and resuspended in 100 µl of 0.1 M Tris buffer (pH 8) (Kjargaard et al., 2002). Protein estimation was done by method of Lowry et al., 1951 using bovine serum albumin as the standard.

2.11.2. Indirect ELISA

The antigen preparation was diluted to a final concentration of 20 µg/ml in 1X PBS. The wells of a microtitre plate were coated with diluted 50 µl of antigen and incubated at 4°C overnight. The coating solution was removed and the plate was washed three times with 200 µl washing buffer (1X PBS containing 0.05% v/v Tween 20). The remaining protein binding sites in the coated wells were blocked by adding 200 µl of blocking buffer, 1% BSA in 1X PBS and incubated for 2 hrs at room temperature. The plate was washed twice with washing buffer. Primary antibody was diluted at three different dilutions i.e. 1:5000, 1:10000 and 1:15000 in blocking buffer and 100 µl was added to each well and incubated for 2 hrs at room temperature. The plate was washed four times with washing buffer. Secondary antibody (HRP conjugated goat anti-rabbit IgG antibody; Bangalore genei, Banglore) was diluted 1:1000, added to wells and incubated for 1-2 hrs at room temperature. The plate was washed four times with washing buffer. The detection was done by adding 100 µl of 1X substrate, TMB/H_2O_2 (Tetramethybenzidine/Hydrogen peroxide). After sufficient color development the reaction was stopped by adding 100 µl of 2M of H_2SO_4. The micro titre plate was placed inside the ELISA plate reader and absorbance was measured at 450 nm.

2.12. Macrophage: Bacteria interactions studied by FACS

2.12.1. THP 1 macrophage cell line culture

The THP1 monocyte cell line was obtained from NCCS, Pune, India. The cell line was maintained in RPMI Medium supplied with 10% FBS and gentamicin (10μg/ml) at 37 °C and 5 % CO_2. The THP1 cells were induced with PMA (200 nM) for 24 hrs to differentiate the monocytes into macrophages. The number of macrophages was counted using Neubauer chamber prior to the Mφ: *E.coli* interaction experiment. The cells were resuspended in RPMI media without antibiotic at the count of 2 X 10^5/ml.

2.12.2. Labelling of bacterial cells with FITC

The bacterial culture was labelled as described by Weingart et al., 1999. The bacteria from overnight cultures were harvested, suspended into phosphate-buffered saline, and adjusted to an OD_{600} of 1.0 i.e. approximately 1 X 10^9 cells / ml. Bacteria were transferred to a microfuge tube, pelleted and resuspended in 1 ml of FITC (1 mg/ml in 50 mM sodium carbonate and 100 mM of sodium chloride, pH 8.0). Bacteria were incubated for 20 min at room temperature, washed three times with 1 X PBS and finally resuspended in 1 X PBS. The labelled bacteria were stored at 4°C and were used the same day for macrophage interaction. The labelling efficiency of the cells was checked on the FACS Calibur.

2.12.3. Interaction of Macrophage: Bacteria and FACS analysis

For interaction of macrophage and bacteria first the standardization was done with three different ratio of Mφ: *E.coli* i.e. 1:50, 1:100 and 1:200 and incubation time of 60 and 120 minutes. The number of Mφ taken was 2 X 10^5 and the number of *E.coli* cells was 1 X 10^7 (1:50), 2 X 10^7 (1:100) and 4 X 10^7 (1:200). The appropriate number of macrophages with FITC labelled *E.coli* cells was incubated for 60 minutes. The media was removed and replaced with fresh RPMI + gentamicin (10μg/ml) and incubated for 30 minutes to kill the unbound bacterial cells. The media was removed and the cells were washed with 1X PBS. The macrophages were then detached from the well by adding cell dissociation solution and cell scrapper. The macrophages were washed with 1X PBS and fixed with 4% paraformaldehyde. The samples were analyzed by FACS on FACS Calibur.

Results and Discussion

3.1 Functional characterization of small RNA RyjA

3.1.1. RyjA as a proposed regulator of superoxide stress

The multifaceted search strategy for novel sRNAs in *E. coli* was conducted by many scientific groups in early years of this decade. In year 2001, RyjA and SraL (psrA 24) (Argaman *et al*., 2001; Wassarman *et al*., 2001) were independently identified in the initial screens for small RNAs in *E.coli*. Later RyjA and SraL were reported to be the identical sRNA (Gottesman, 2004). RyjA, positioned at 4275950 - 42756089 on minus strand of *E. coli* genome and expressed from a σ^{70} promoter, is predominantly present in late stationary phase. The *ryjA* gene is flanked by two reading frames, *sox*R and *yjc*D, both of which are transcribed in the direction opposite to that of *ryj*A (Argaman *et al*., 2001; **Fig. 3.1.1**).

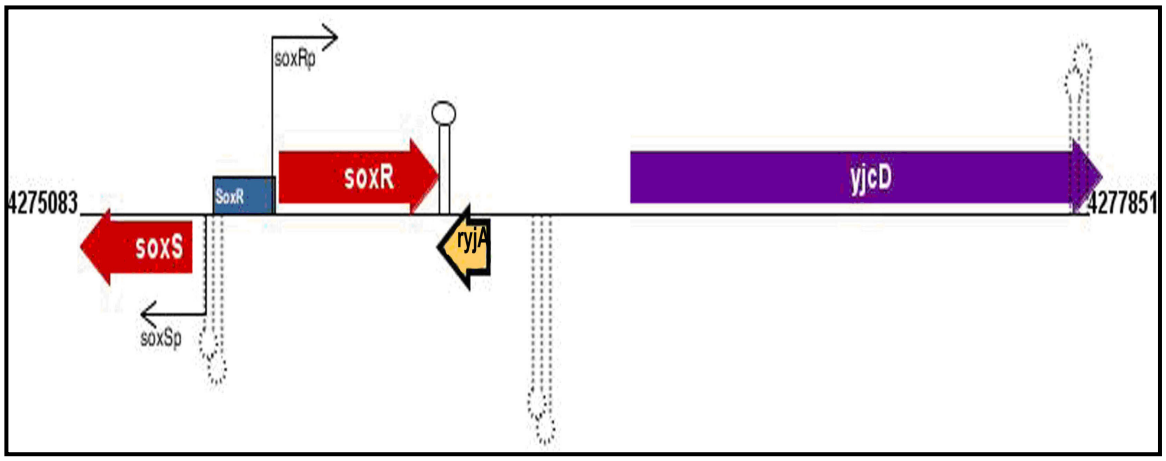


Fig. 3.1.1: Schematic representation of the *ryjA* and its neighbouring genes on the *E. coli* K12 genome (www.ecoliwiki.net).

A transcriptional terminator consisting stretches of uridines at both ends of an inverted repeat functions bi-directionally for both *ryj*A and *sox*R **(**Argaman *et al*., 2003; **Fig.3.1.2**). The *soxR* and *ryjA* genes are convergently transcribed from different promoters leading to transcripts with 3' overlapping ends. Thus there is a high possibility of RyjA sRNA base pairing with the 3' complementary end of *soxR* transcript and acting as a *cis*-regulator.

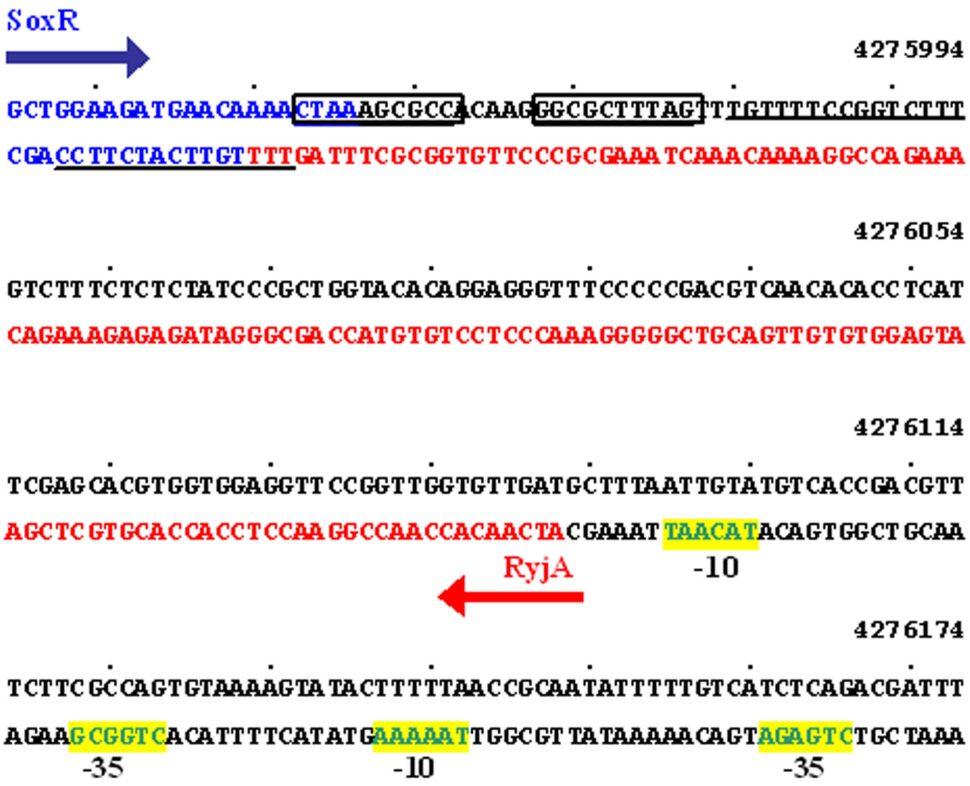


Fig. 3.1.2: The genomic location of *ryjA* and its bidirectional terminator: The DNA sequence in red color indicates *ryjA* gene encoding RyjA sRNA, whereas the sequence in blue color indicates *soxR* gene encoding SoxR mRNA. The inverted repeat sequences in the top and bottom strands are represented in boxes and indicate the bidirectional terminator. The stretches of uridines on both side of the terminator are underlined. The -10 and -35 hexamers of the σ^{70} consensus of two promoters, the far-left and far-right putative promoters are shown (yellow highlight). The numbering indicates the position in the *E. coli* genome database. ORF *yjcD* is located downstream of the area shown and starts at 4276059 **(**Argaman *et al*., 2001).

3.1.2. Role of *E. coli* S*oxRS* regulon under super oxide stress

In *E. coli*, the *soxRS* (superoxide response) regulon mediates an oxidative stress response that protects the cells against the superoxide anion radical (O_2^-), nitric oxide and redox cycling reagents, such as paraquat (methyl viologen), plumbagin and phenazine methosulfate (Walkup *et al*., 1989; Greenberg *et al*., 1990, Tsaneva *et al*., 1990). The *soxRS* regulon is a two component regulon consisting of SoxR and SoxS as sensor and regulator respectively. The SoxR protein occurs in reduced and oxidized forms, the latter acting as the transcriptional activator of *soxS* transcription (Wu and Weiss, 1991; Amabile-Cuevas and Demple, 1991).

SoxR (17 KDa, dimer) is member of the Mer family of metal binding transcriptional factors that contains [2Fe-2S] centers per monomer and is produced constitutively at a low level of ~ 100 molecules per cell. When the cell is exposed to sub lethal doses of superoxide generating agents, the [2Fe-2S] center of SoxR is oxidized $[2Fe\text{-}2S]^{2+}$, and the SoxR activates the transcription of the only known target gene, *soxS*. However, upon removal of oxidative stress condition the SoxR is reduced by one electron $[2Fe\text{-}2S]^{1+}$, the ability to activate *soxS* transcription is lost (Ding *et al*., 1996; Gaudu and Weiss, 1996). The electroparamagnetic resonance analysis of SoxR and measurement of re-reduction kinetics suggested that the products of two genetic loci (*rsx* and *rse*) are necessary for maintaining the reduced state of SoxR (Koo *et al*., 2003). The reduced and oxidized SoxR have equal affinity to bind to its DNA site but only oxidized SoxR triggers the *soxS* transcription (Hidalgo *et al*., 1998) (**Fig. 3.1.3)**.

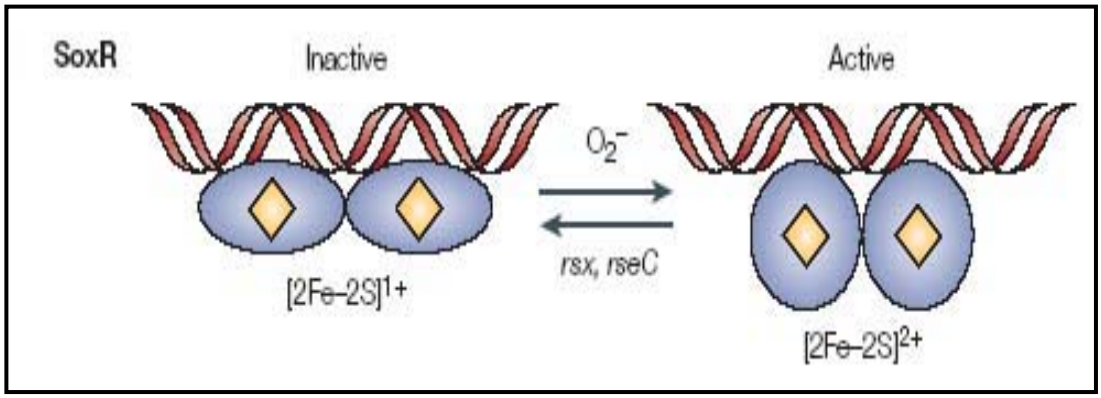


Fig. 3.1.3: Activation of SoxR by superoxide anion (O_2^-): The inactive SoxR is activated by oxidation of its [2Fe±2S] cluster while the *rsx* and *rseC* gene products constitute a reducing system for maintaining SoxR in its reduced inactive state (Koo *et al*., 2003).

The activated SoxS, a simple transcriptional activator of the AraC family increases the transcription of the other genes of the regulon (Wu and Weiss 1991; Amabile-Cuevas, and Demple, 1991). The known activated genes are *sodA* (encoding Mn-superoxide dismutase), *fpr* (NADPH-ferredoxin oxidoreductase), *micF* (antisense RNA, repressor of OmpF translation), *ribA* (cyclic GMP hydrolase), *inaA* (unknown function, *fidA* and *fldB* (flavodoxins A and b), *nfo* (endonuclease IV), *marRAB* (multiple antibiotic resistance operon) *nsfA* (also called mdaA, a nitroreductase), *zwf* (glucose-6-phosphate dehydrogenase), *fur* (an iron-binding repressor of iron uptake), *fumC* (fumarase C), *acnA* (aconitase), *tolC* (outer membrane protein), *acrAB* (drug efflux pump), and *rimK* (a modifier of ribosomal protein S6) (Pablo, *et al*., 2001)

(**Fig. 3.1.4)**. The diversity of genes activated by SoxS illustrates the variety of cellular defence mechanisms against oxidative stress which damages virtually all biological macromolecules. Antioxidant mechanisms include the scavenging of reactive species (*sodA*, *ahpCF*), synthesis of reducing species (*acnA*, *zwf*), repair of oxidative damage (*nfo*, *fpr*), drug efflux (*acrAB*, *tolC*), reduction of cell permeability (*micF*), and replacement of redox-sensitive ribozymes by redox-resistant isozymes (*fumC*). However a genome wide transcriptional profiling of the *E. coli* responses to super oxide oxidative stress suggests a total of 112 genes were modulated when stressed with paraquat (Pablo, *et al*., 2001).

Blanchard *et al*., (2007) employed a time series microarray design and identified a total of 138 genic regions, including several transcription factors and putative sRNAs regulated through the SoxRS signalling pathway within 10 minutes of paraquat (PQ) treatment. The SoxRS-dependent genes were shown to include four known transcriptional regulators (Rob, MarA, Fur and OmpR), a sigma factor (RpoH), five genes annotated as putative transcription factors (YhcA, ChaC, FrvR, YbaO and YbdO), and three known regulatory sRNAs (MicF, MicC and RydB). Thus, SoxS has been interpreted as global regulator that modulates the expression of other regulators. The SoxRS-independent response included members of the OxyR, CysB, IscR, BirA and Fur regulons (Blanchard *et al*., 2007).

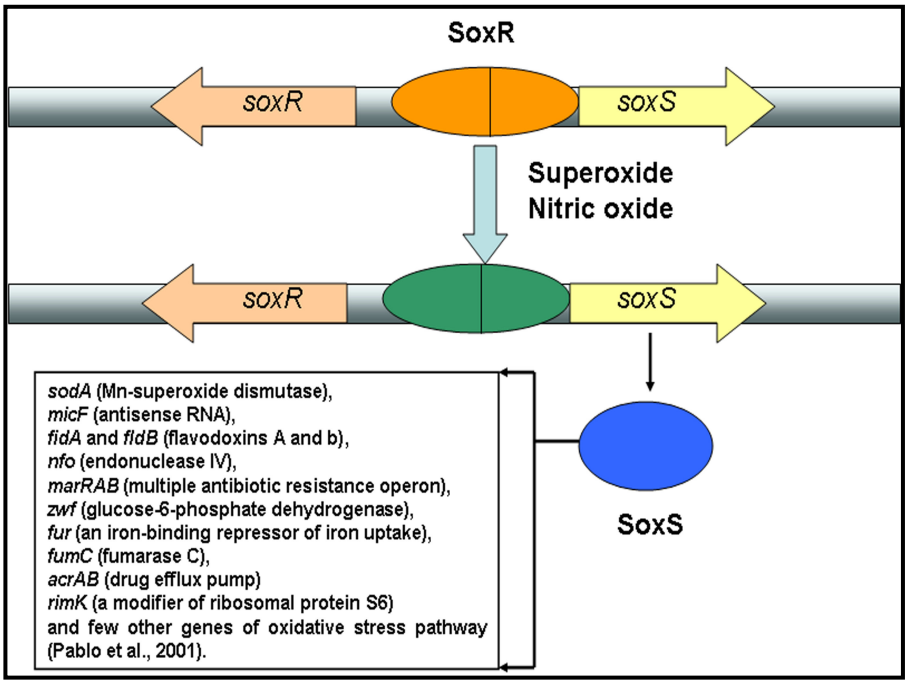


Fig. 3.1.4: Schematic representation of *E. coli* SoxRS regulon (Pablo *et al*., 2001).

3.1.3. Results of bioinformatics analysis for RyjA targets identification

3.1.3.1. BLAST search

The target genes for the uncharacterized *E. coli* sRNAs were identified by most simple and straight bioinformatics tool BlastX which finds regions of sequence similarity and yields functional and evolutionary clues about the structure and function of the novel sequence. The BlastX search for RyjA indicated a match only with *E. coli* SoxR protein of *soxRS* regulon. The >18 bp complementarity of RyjA at its 3' end to that of SoxR suggested RyjA to be a *cis*-encoded regulatory sRNA putatively regulating SoxR through base pairing interactions. Such previously reported sRNAs that possess *cis* regulatory role include *E. coli* GadY (Opdyke *et al*., 2004) and SymR (Kawano *et al*., 2007).

3.1.3.2. Predicted Secondary structure of RyjA

The secondary structure of RyjA was analysed by computer folding program Sfold (Ding *et al*., 2004). The structure exhibited three distinct stem-loops (1, 2 and 3) wherein stem loop 3 consisted of long stretches of uridines making it unstable and hence accessible for complementary base-pair interactions with target mRNA (**Fig. 3.1.5**). The secondary structure of RyjA was also predicted from other programs such as Mfold and RNAfold and was found to be similar.

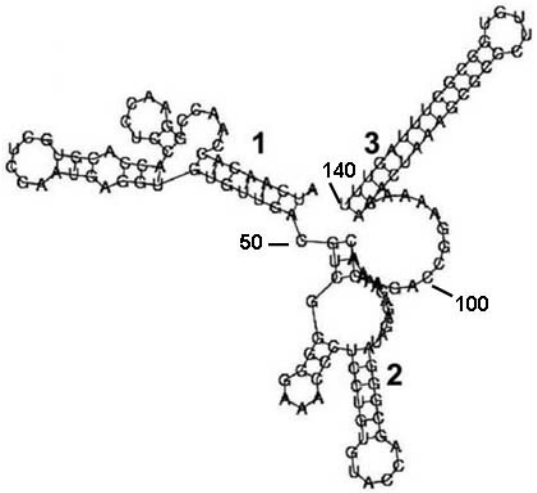


Fig. 3.1.5: RyjA secondary structure: Predicted secondary structure of RyjA based on Sfold program. 1, 2 and 3 indicate stem-loop 1, 2 and 3 respectively.

3.1.3.3. TargetRNA program

TargetRNA program is based on characteristic features of known antisense-target interactions and effectively predicts mRNA targets of base pairing sRNAs. It calculates optimal hybridisation scores for sRNA-target RNA hybrids and gives a list of candidate mRNAs (Tjaden *et al*., 2006). The RyjA sequence when searched for target mRNAs using TargetRNA program predicted four targets (**Table 3.1.1**). However all the predicted targets were either hypothetical or putative proteins whose functions were difficult to analyze.

Table 3.1.1: List of predicted targets of RyjA sRNA by TargetRNA program

Target gene product	**sRNA:Target interaction**	**Alignment score**	**P value**
YgeZ : Hypothetical protein	**mRNA -1 UUUGGGAGGCCGCUUGGCCUCCCCUUAU -28** **||||||||| |:| ||| || ||:|||** **sRNA 59 AAACCCUCCUGUGUACC--AGCGGGAUA 84** **(stem loop 2)**	-70	0.0020
YhhJ: Inner membrane protein with ABC trans-membrane type 2 domain	**mRNA -1 GGGAGGACAAAAAAGUCGUCGUCCUAU -27** **||||||||| | | ||||:|||||** **sRNA 62 CCCUCCUGUGU----ACCAGCGGGAUA 84** **(stem loop 2)**	-66	0.0041
YgbN: Gluconate transporter; inner membrane protein	**mRNA 17 UUACAUUA-ACACCUGUA---CCCUUUGGGA -10** **|||| ::| ||| |||:| ||||||||||** **sRNA 35 AAUGAGGUGUGUUGACGUCGGGGGAAACCCU 65** **(stem loop 1)**	-65	0.0049
YhfK: Hypothetical protein	**mRNA 18 UUAGUCAGACGCGG-UGUAGCCCCCUUUG -10** **||| || ||:|:: ||:|||||||||** **sRNA 35 AAUGAGG-UGUGUUGACGUCGGGGGAAAC 62** **(stem loop 1)**	-65	0.0049

3.1.4. Analysis of the effect of multicopies of RyjA on growth physiology and SoxRS expression

3.1.4.1. Construction of RyjA over expressing plasmid (pRyjA)

The RyjA sRNA gene was amplified from *E. coli* KL16 with RyjAFP and RyjARP primers (**Fig. 3.1.6. a**), cloned and over expressed under the strong and inducible *lac* promoter of the multicopy vector pNEB206A of USER (uracil-specific excision

reagent) Friendly cloning kit (NEB). The cloning in the linear vector pNEB206A was carried out by using an insert having complementary adapters to the 8 bp 3' overhangs in the vector (vector map and cloning strategy given in Appendix). The recombinants obtained were confirmed first by digesting with HindIII and EcoRI where an insert release of 240 bp was obtained (**Fig. 3.1.6. b**) and later by sequencing (Appendix). The RyjA over expressing plasmid was named as pMNC10.

The effect of RyjA multicopies on SoxR expression was studied by measuring the expression of the only known direct target *soxS* in *E. coli* BW1157 strain that bears a λ mediated integration of an additional copy of *soxS* as *soxS*:*lacZ* fusion and hence ampicillin resistant. The pMNC10 was modified to include the tetracycline resistance gene (2.1 kb) as a selection marker for transformation into ampicillin resistant BW1157. The TetR gene along with its promoter from pBR322 was excised by SspI and PvuII (**Fig. 3.1.7. a**) and ligated in SacI and SspI digested pMNC10 replacing the ampicillin resistance gene. The recombinants obtained i.e. pRyjA were thus tetracycline resistant but ampicillin sensitive. The pRyjA when digested with HincII gave two bands of 2654 bp and 2194 bp (**Fig. 3.1.7. b**) as it cuts at 47th nt in *ryjA* and at 651 nt in tetracycline resistance gene.

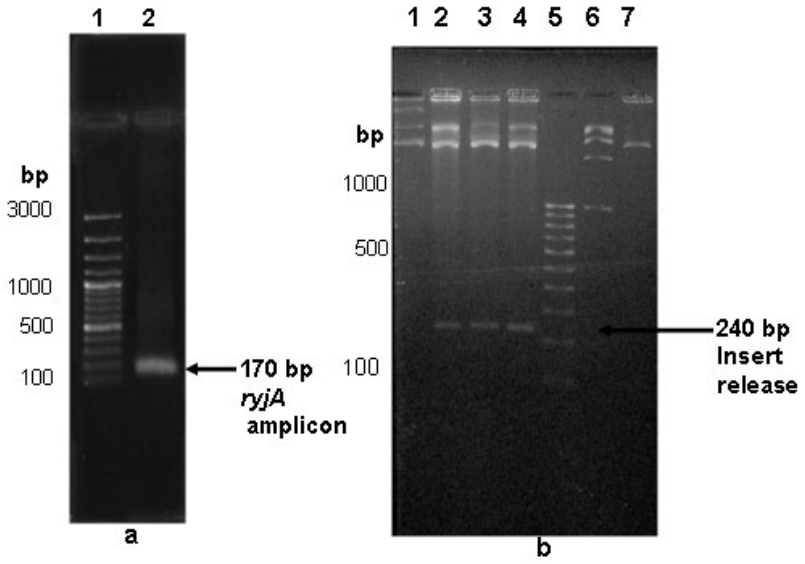


Fig. 3.1.6: Construction of RyjA over expressing plasmid: **(a) Amplification of *ryjA* by high fidelity X-Taq DNA polymerase**. Lanes: 1, 100 bp ladder; 2, ~ 170 bp *ryjA* amplicon. **(b) Confirmation of pMNC10 by restriction analysis**: Lanes: 1, Undigested pMNC10; 2, 3, & 4, recombinants digested with HindIII and EcoRI; 5, 100 bp ladder; 6, Undigested pNEB206A; 7, pNEB206A digested with HindIII and EcoRI

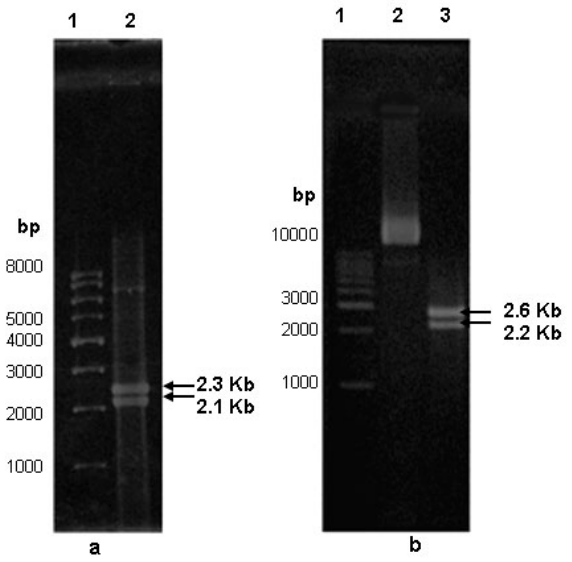


Fig. 3.1.7: Insertion of tetracycline marker gene in pMNC10:

(a) Excision of tetracycline gene from pBR322: Lanes: 1, 1 Kb ladder; 2, pBR322 digested with SspI and PvuII released 2.1 kb tetracycline gene.

(b) Verification of pRyjA by restriction analysis: Lanes: 1, 1 Kb ladder; 2, Undigested pRyjA; 3, pRyjA digested with HincII.

3.1.4.2. Construction of the vector (pNEB206AT)

The use of pRyjA necessitated the construction of an isogenic control vector lacking *ryjA*. Therefore the *ryjA* from pRyjA was removed by digestion with PstI and SacI. The vector backbone was eluted, end-filled by T4 DNA Polymerase and religated to yield a control vector pNEB206AT (**Fig. 3.1.8. a**). The construct was confirmed by digesting with HincII which now only has one site due to the removal of *ryjA* (**Fig. 3.1.8. b**). Only a single band of linearized plasmid was obtained suggesting that it lacked *ryjA*.

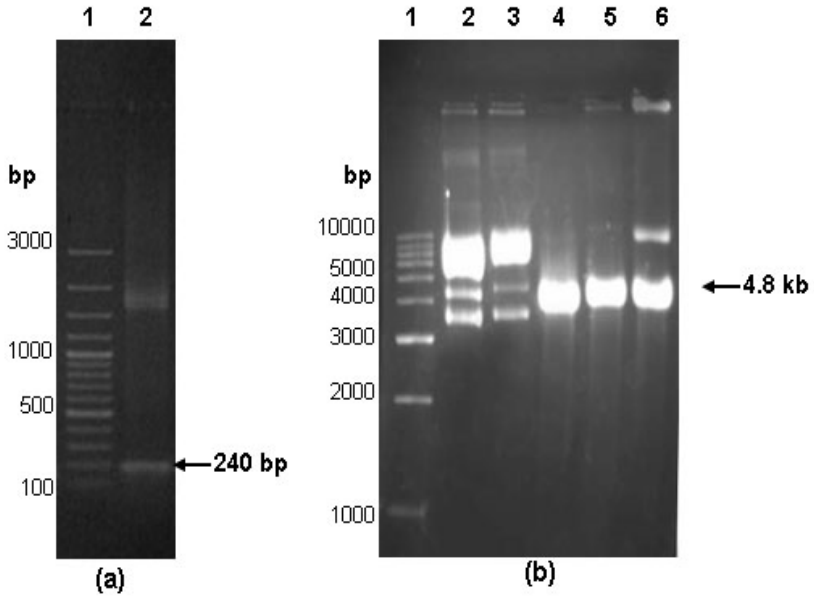


Fig. 3.1.8: Construction of pNEB206AT vector: (a) Digestion of pRyjA: Lanes: 1, 100 bp MW marker; 2, pRyjA digested with PstI and SacI showing an insert release of *ryjA* (200 bp).

(b) Confirmation of pNEB206AT vector by restriction analysis. Lanes: 1, 1 kb MW marker; 2-3, undigested pNEB206AT; 4-6, pNEB206AT digested with HincII.

3.1.5. Effect of multicopies of RyjA

3.1.5.1. Growth physiology and SoxRS expression

Since SoxR activates transcription of *soxS,* any regulation of SoxR can be studied by measuring the expression of the *soxS′-′lacZ* fusion. To study the high copy number effect of RyjA, the plasmids pRyjA and pNEB206AT were introduced into the strains *E. coli* BW1157 and *E. coli* BW1043 which respectively bear translational fusions, *soxS′-′lacZ* and *soxR′-′lacZ.* These strains are derivative of *E. coli* GC4468 strain and have λ mediated integration of *soxS′-′lacZ* or *soxR′-′lacZ* element at the *attλ* site in addition to the chromosomal copy of SoxR (Gaudu and Weiss, 2000). The effect of multicopies of RyjA on SoxR expression was estimated by SoxR-dependent induction of *soxS'-'lacZ* fusion. The presence of RyjA in pRyjA in multiple copies resulted in ~7 fold inhibition of *soxS′-′lacZ* expression in BW1157 (**Fig. 3.1.9**) when induced with IPTG and PQ. The decreased expression of *soxS'-'lacZ* fusion could be a consequence of the RyjA mediated reduction of SoxR which in turn could be due to increased levels of RyjA. The SoxR is probably down regulated by its base pairing with RyjA present in multicopies.

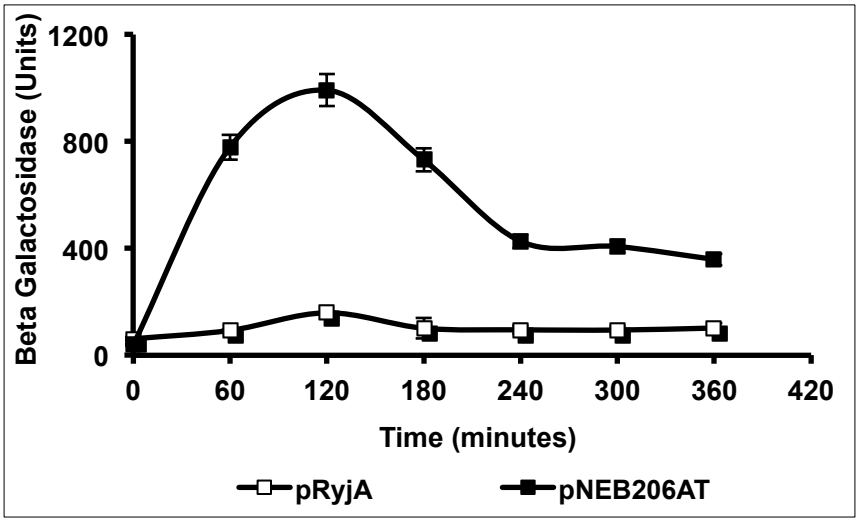


Fig. 3.1.9: Effect of RyjA overexpression on expression of *soxS′-′lacZ* translational fusion: Overnight culture of ***soxS′-′lacZ*** (pRyjA) & (pNEB206AT) were inoculated in LB (1% v/v). The *ryjA* expression was induced by addition of IPTG (20 μg ml^{-1}) and the oxidative stress by paraquat (50μM) at an optical density of ~ 0.4 at 600. β galactosidase activity measured reflects *soxR* expression. Error bars indicate standard deviation in three independent experiments.

The effect of multicopies of RyjA on *soxR'-'lacZ* expression was studied in *E. coli* BW1043. SoxR is a negative autoregulator and maintains a constant level under oxidative stress. Therefore in presence of multicopies of RyjA the expression of

soxR'-'lacZ was expected to be identical to that of the control strain. However a reduction of 3.5 fold in *soxR'-'lacZ* expression was observed in pRyjA which based on the observation of regulation of SoxR expression is unclear. (**Fig. 3.1.10**).

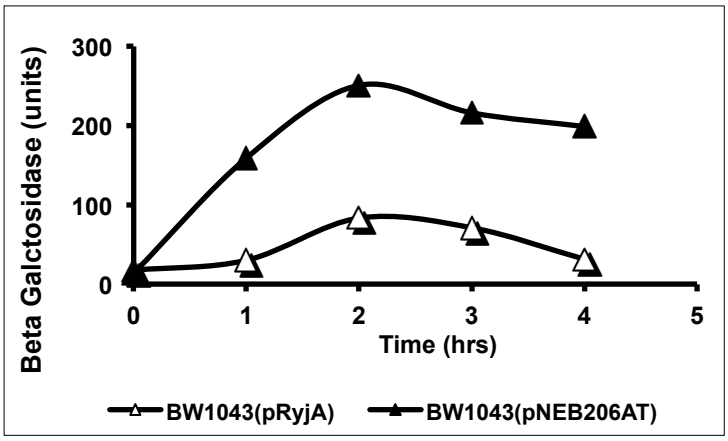


Fig. 3.1.10: Effect of RyjA overexpression on expression of *soxR´-´lacZ* translational fusion: Overnight culture of BW1043 (pRyjA) & BW1043 (pNEB206AT) were inoculated in LB (1% v/v). The *ryjA* expression was induced by addition of IPTG (20 µg ml^{-1}) and the oxidative stress by paraquat (50µM) at an optical density of ~ 0.4 at 600.

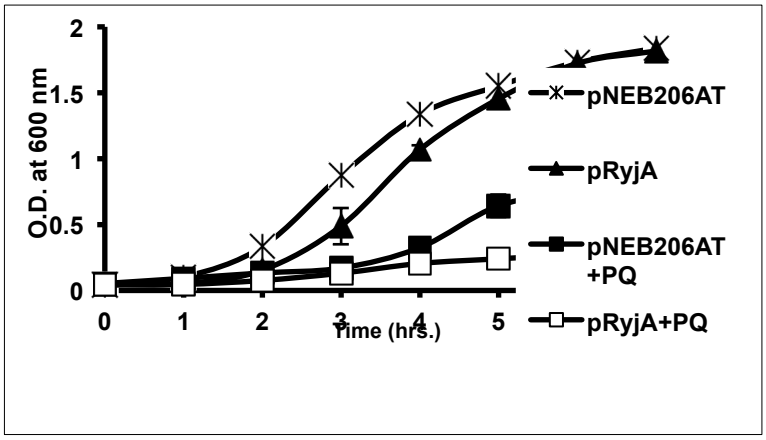


Fig. 3.1.11: Influence of RyjA overexpression on cell growth under oxidative stress. Overnight cultures of ***soxS´-´lacZ*** (pRyjA) and (pNEB206AT) were inoculated in LB (1 % v/v) and the *ryjA* expression was induced by addition of IPTG (20 µg ml^{-1}). Two parallel cultures were set and one set was induced for oxidative stress with paraquat (100µM) and grown at 37ºC, 120 rpm. The growth was measured at OD_{600}. The data represented is mean of three experiments.

Expression of RyjA from multicopy plasmid pRyjA also resulted in poor growth of the organism when exposed to oxidative stress (**Fig 3.1.11**) as compared to the isogenic vector control (pNEB206AT). In comparison, both the cultures exhibited similar growth pattern in the absence of paraquat. The effect of oxidation stress was marked in the RyjA over expressing strain and no net growth was observed. However the RyjA overexpressing culture had an initial lag probably because of the presence of the multicopy plasmid expressing the small RNA.

3.1.5.2. Alterations in antibiotic resistance phenotype

The activation of the *soxRS* regulon with PQ treatment or constitutive *soxRS* mutations [called *soxR* (con)] have been reported to increase resistance to ampicillin, nalidixic acid, chloramphenicol, and tetracycline two- to fourfold (Chou *et al*., 1993). The effect of increased dose of antibiotic ampicillin in *soxS′-′lacZ* (pRyjA) was examined. The culture in presence of multi copy RyjA exhibited poorer resistance to ampicillin (500 µg ml^{-1}) when compared to the control (**Fig. 3.1.12**) indicating an indirect effect of RyjA probably resulting from low SoxR levels.

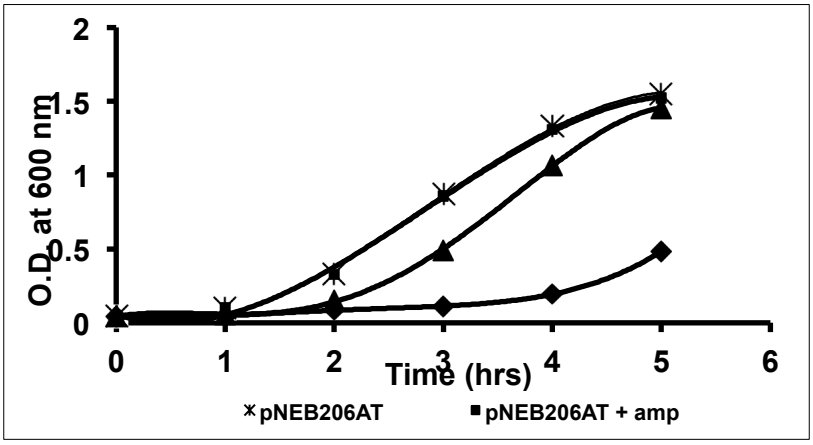


Fig 3.1.12: Effect of RyjA over expression on cell growth under ampicillin antibiotic stress. Overnight cultures of BW1157 (pRyjA) and BW1157 (pNEB206AT) were inoculated in LB (1 % v/v) and the *ryjA* expression was induced by addition of IPTG (20 µg ml^{-1}). Two parallel cultures were set, with 100 µg ml^{-1} and 500 µg ml^{-1} ampicillin concentrations and grown at 37ºC, 120 rpm. The growth was measured at OD_{600}. The data represented is mean of three experiments.

3.1.5.3. RyjA overexpression results in reduced SOD activity

The superoxide dismutase (SOD) activity, one of the several functions regulated by the SoxRS system, was somewhat different under *ryjA* over expression. The SOD activity in cells was measured by the percentage inhibition of the auto oxidation of pyrogallol. A 32% inhibition of auto oxidation was obtained when 780 µg crude cell protein of pNEB206A (IPTG induced) was used. The inhibition by SOD activity with similar amount of cell protein from pRyjA (IPTG induced) was 20% indicating increased autooxidation and therefore reduced SOD activity than the control strain (**Fig 3.1.13**).

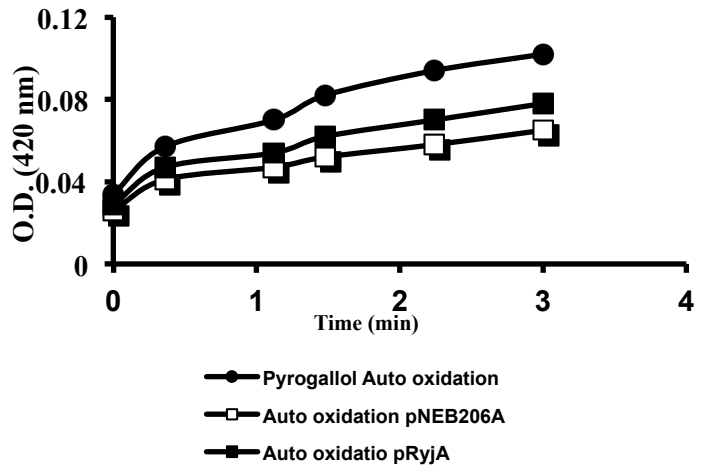


Fig. 3.1.13: Influence of *ryjA* over expression on Super oxide dismutase (SOD) activity: The SOD activity was estimated in *E. coli* DH5α (pNEB206A) and *E. coli* DH5α (pRyjA). All the cultures were grown overnight in LB with IPTG (20 μg ml^{-1}) and paraquat (50μM) at 37ºC, 120 rpm. The lysates were obtained by sonication. The lysate of the culture was added to the 1ml of assay mixture containing pyrogallol and the inhibition of autooxidation of pyrogallol was compared.

3.1.6. Analysis of effect of RyjA disruption and modifications

3.1.6.1. Construction of RyjA disrupted mutant strain (RyjADM)

The chromosomal *ryjA* was disrupted by inserting kanamycin resistance gene. The kanamycin resistance cassette (1.7 kbp) was amplified by KanR and KanF primers (**Fig. 3.1.14. a**) from pBBRMCS-2 (Kovach *et al*., 1994) and cloned into pMNC10 which was linearized with HincII. The enzyme HincII cuts at 47^{th} nucleotide without disturbing the transcription terminator region of *ryjA* predicted to be involved in the regulation of SoxR expression (**Fig. 3.1.15**). The recombinant plasmid pRyjAKan (*ryjA* disruption plasmid) obtained were selected on LA plates containing kanamycin (40 μg ml^{-1}) and were confirmed by PCR amplification that yielded an amplicon of 1.9 Kbp using RyjA specific primers. The additional band of 170 bp results from the chromosomal copy (**Fig. 3.1.14. b**). The recombinants were further confirmed by digestion using restriction enzymes HindII and EcoRI and the insert release of 1.9 Kbp (**Fig. 3.1.14.c**).

The disrupted *ryjA* sequence was integrated by homologous recombination into the chromosome of *E. coli* DY330 using λ recombinase to yield the *ryjA* disrupted strain. For this, the KmR-disrupted *ryjA* gene was amplified and the linear DNA was

electroporated into freshly prepared recombination proficient *E. coli* DY330 electrocompetent cells. The *E. coli* DY330 strain is a recombination proficient strain containing three λ recombinase genes *exo*, *bet* and *gam* all under the control of a temperature sensitive promoter which gets turned on at 42°C (Yu *et al*., 2000). The kanamycin resistant *ryjA* disrupted mutants were also confirmed by PCR using RyjA primers. The amplicon of ~140 bp was obtained from wild type while the *ryjA* disruptant strain showed an amplicon of 1.9 Kbp indicative of *ryjA* sequences flanking the kanamycin resistance cassette and hence disrupted (**Fig. 3.1.14.d**). The clones were further confirmed by Northern blot.

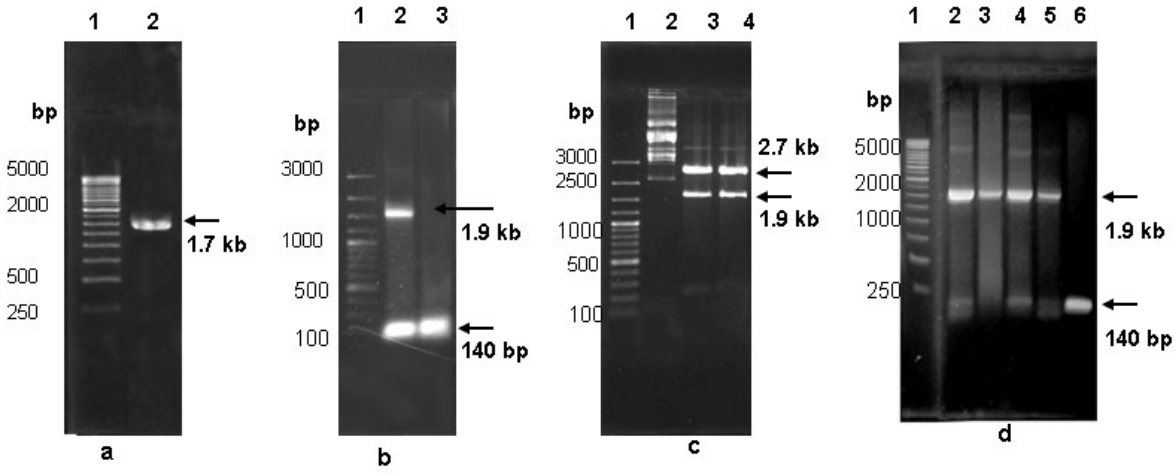


Fig. 3.1.14: Disruption of chromosomal *ryjA*: (a) Amplification of kanamycin resistance gene using high fidelity X-taq polymerase: Lanes: 1, 250 bp MW marker; 2, Kanamycin resistance cassette amplicon from pBBRMCS-2 plasmid DNA. **(b) Confirmation of pRyjAKan by PCR using RyjAFP and RyjARP primers**: Lanes: 1; 100 bp ladder; 2, amplicon with pRyjAKan DNA as template DNA; 3, amplicon with pMNC10 as template DNA. **(c) Verification of pRyjAKan by restriction analysis:** Lanes 1; 100 bp MW marker; 2, undigested pRyjAKan; 3 & 4, pRyjAKan digested with HindIII and EcoRI. **(d) Confirmation of *ryjA* disruption in *ryjA* disrupted strains by PCR using RyjAFP and RyjARP primers:** Lanes 1; 100 bp MW marker; 2-5, *ryjA* amplified from genomic DNA of *ryjA* disrupted strains, *E. coli* RyjADM1, RyjADM2, RyjADM3 and RyjADM4; 6, *ryjA* amplified from *E. coli* DY330 genomic DNA.

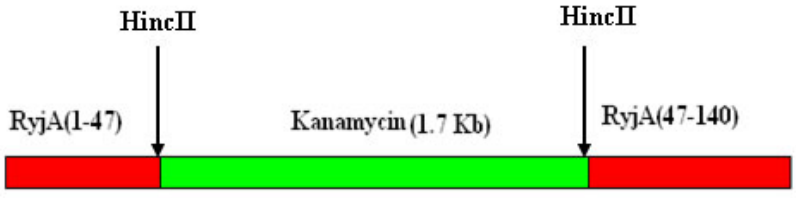


Fig. 3.1.15: Schematic presentation of Kanamycin resistance cassette within *ryjA*.

3.1.6.2. Disruption of chromosomal *ryjA* and the consequent physiology

In order to analyze the effect of the complete absence of RyjA transcripts, disruption in the chromosomal *ryjA* was created. Under oxidative stress, the *ryjA* disruption mutant and the isogenic DY330 exhibited cell doubling times of approximately 2.4 hrs during the initial logarithmic growth (~6 hrs). Thereafter these strains exhibited a drastic difference in growth rate with a doubling time of 14.1 hrs for the DY330 strain compared to the 5.1 hrs of RyjADM when calculated for growth at 10 and 15 hrs (**Fig. 3.1.16**).

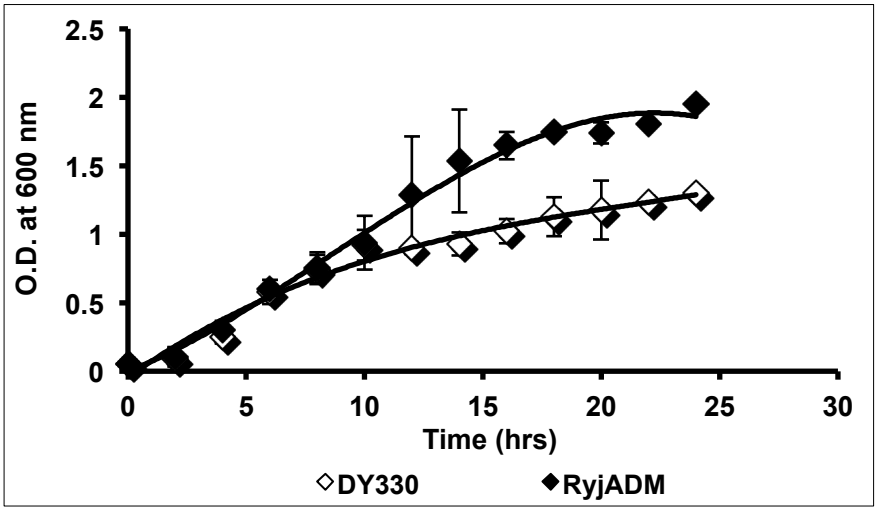


Fig. 3.1.16: Influence of RyjA disruption of *ryjA* on the cell growth under oxidative stress: Overnight cultures of *E. coli* DY330 and *E. coli* RyjADM were inoculated in LB (1% v/v) and induced by IPTG (20 µg ml^{-1}) and paraquat (50 µM) and grown at 37ºC, 120 rpm. The growth was measured at OD_{600}. The data represented is mean of three experiments.

Next to test the effect of disruption of chromosomal *ryjA* on *soxS'-'lacZ* expression, the plasmid pWB53 bearing *soxS'-'lacZ* fusion was transformed in lac^-, $ryjA^+$ (*E. coli* DY330) and $ryjA^-$ (*E. coli* RyjADM) strains. The β galactosidase activities were measured in stationary phase. The specific activities of β galactosidase in $ryjA^+$ and $ryjA^-$ strain were similar (**Fig. 3.1.17**). Such an observation could be expected and justified when negative effect of the single copy *ryjA* regulator is nullified by the expression of target gene from a multiple copy plasmid. Therefore the difference in SoxRS expression in $ryjA^+$ and $ryjA^-$ were quantitated by real time PCR.

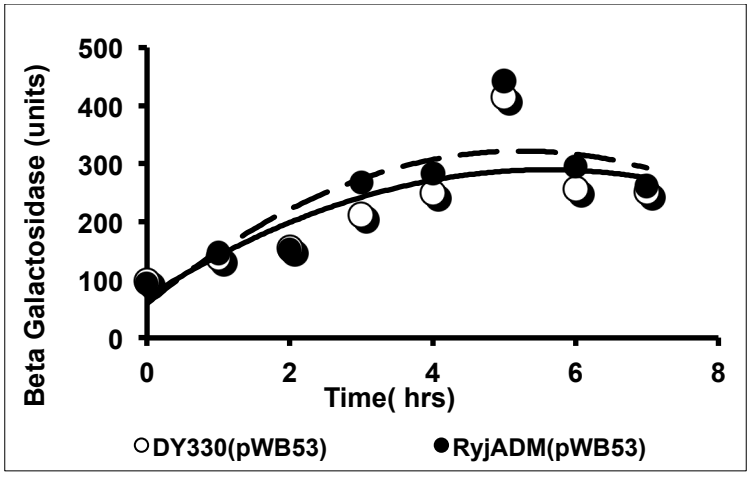


Fig. 3.1.17: Analysis of *ryjA* disruption on the expression of *soxS′-′lacZ* translational fusion: The cultures RyjADM (pWB53) and DY330 (pWB53) were grown for 8 hours. β galactosidase activity was measured from 8 hrs till 22hrs.

3.1.6.3. Construction of plasmids deletion derivatives of RyjA (pRyjAΔ2 and pRyjAΔ3)

Since the RyjA was cloned (pRyjA) in a modified derivative of pNEB206A (linear vector), tetracycline resistant, it was essential to generate a vector backbone from the plasmid for all further cloning. The pRyjA was digested to replace the RyjA with the amplicons of *ryjA* deletion or substitution derivatives.

Prediction of secondary structure of RyjA by *Sfold* (Ding *et al*., 2004) showed three stem loops 1, 2 and 3 (**Fig. 3.1.18 b**). Plasmids with RyjA carrying independent deletion in each stem were constructed for assessing the stem domains essential for RyjA function. Deletion of individual stems of RyjA in silico revealed that the deletion of one loop did not alter the secondary structure of the remaining two loops in the predicted structure of RyjA.

The RyjA deletion derivatives were generated by PCR extension of overlapping complementary primers designed to lack sequences corresponding to one stem in each construct. The stem 2 deletion derivative lacked sequences at 49-95 nt while stem 3 deletion derivative had sequences removed at 110bp-140bp (**Fig.3.1.18 c & d).** Efforts to generate stem1 deletion did not yield any clones. Oligonucleotide primers for the RyjA stem 2 and stem 3 deletion mutants were tagged with PstI and SacI sequences at their 5' ends. The amplified fragments **(Fig. 3.1.19 a**) were cloned into PstI and SacI digested pNEB206AT vector. All the clones were confirmed by insert

release (**Fig. 3.1.19 b & c**) and subsequent sequencing by M13 primers at Bangalore Genei, India (Appendix). The pRyjAΔ2 recombinants were digested with Hpy991 restriction enzyme which has lost its one site upon removal of stem 2. When digested, the true recombinants do not contain a 350 bp fragment while the false positives had an extra fragment of 350 bp along with other bands.

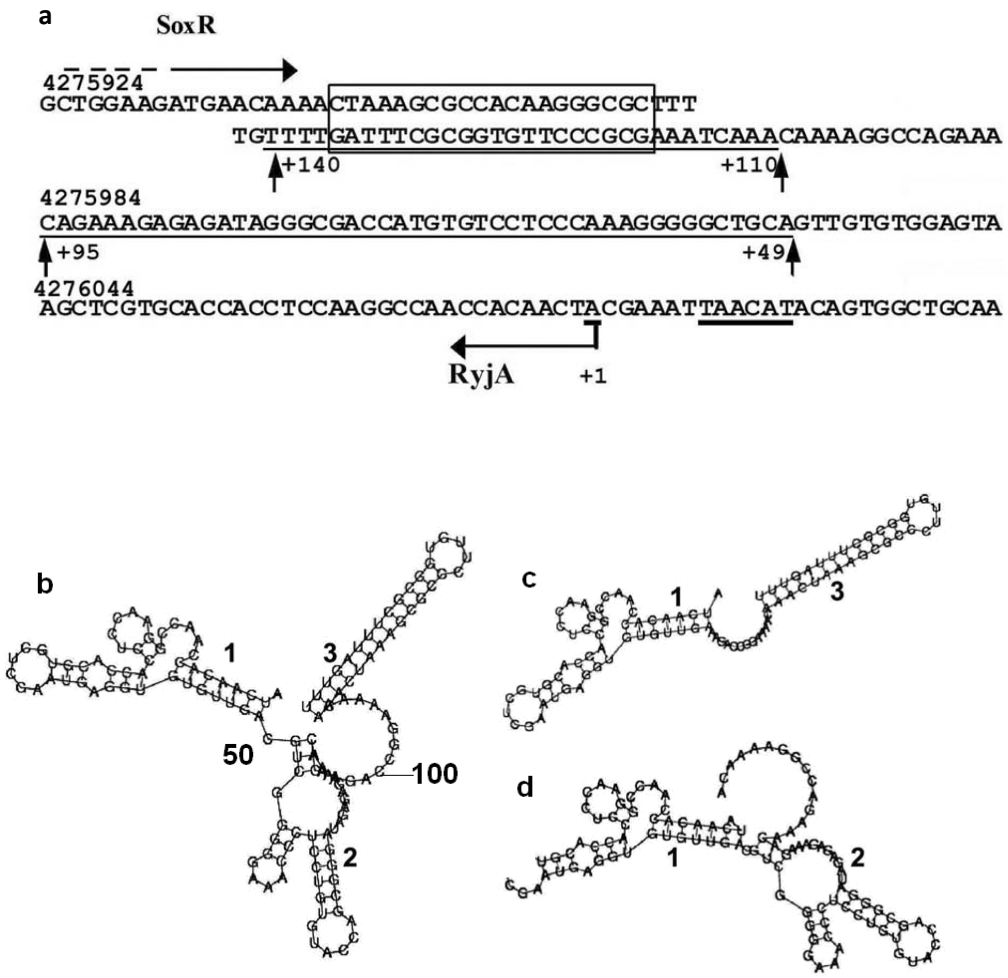


Fig. 3.1.18: Nucleotide sequence and secondary structures of *ryjA* and derivatives. (a) RyjA nucleotide sequence showing 3' overlap (boxed) to SoxR. Nucleotides deleted in pRyjAΔ2 (49-95) and pRyjAΔ3 (110-140) are underlined. Numbers on the left indicate positions in *E. coli* K12genome. (**b**), (**c**) and (**d**) are predicted secondary structures of *ryjA, ryjA* Δstem2 and *ryjA* Δstem3 respectively.

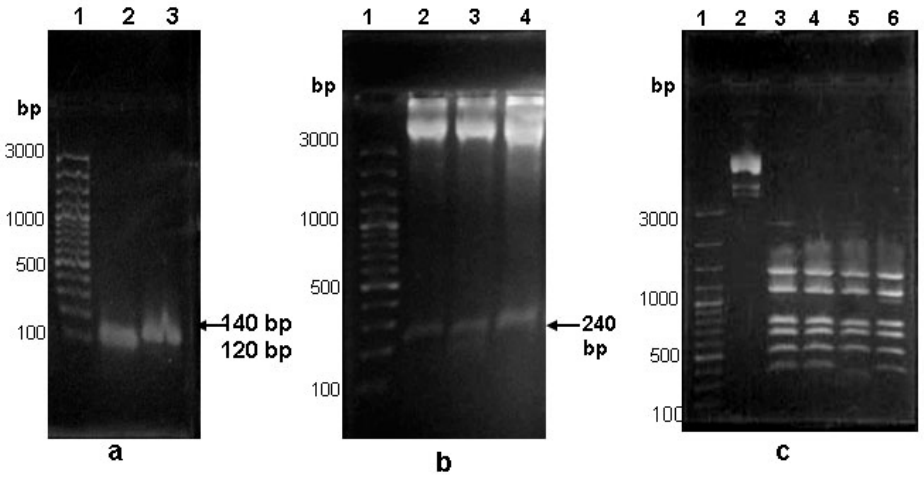


Fig.3.1.19: Construction of RyjA deletion mutants, pRyjAΔ2 and pRyjAΔ3: (a) Amplification of RyjA2 and RyjA3 by appropriate primers: Lanes: 1, 100 bp MW marker; 2, RyjA2 amplicon (120 bp); 3, RyjA3 amplicon (140 bp). **(b) Confirmation of pRyjAΔ2 recombinants by insert release.** Lanes: 1, 100 bp MW marker; 2-3, pRyjAΔ3 recombinants digested with PstI and SacI indicating an insert release of 240 bp; 4, pRyjAΔ2 digested with PstI and SacI indicating an insert release of 240 bp. **(c) Digestion of pRyjAΔ2 recombinants with Hpy99I:** Lanes: 1, 100 MW marker; 2, undigested pRyjAΔ2; 3-6, recombinants digested with Hpy99I; Lane 5 does not show a high molecular weight band of 350 bp due to loss of Hpy991 site by absence of stem 2.

3.1.6.4. Construction of RyjA stem 3' substitution mutation

Deletion of RyjA stem-3 is expected to result in poor termination of transcripts. However a mutant derivative of a *ryjA* gene carrying a substituted artificial terminator that lacks complementarity to *soxR,* yet bears structural similarity to loop-3 would express a RyjA with the 3 stem loops structurally identical to that of the wild type *ryjA* and would have no regulatory effects on SoxR. Single stranded oligonucleotides with 3' complementary overlap and corresponding to 5' end of *ryjA* and a 3' alternate terminator of desired modification (**Fig.3.1.21 a**.) were used as forward (RyjAS3FP) and reverse (RyjAS3RP) primers respectively, for annealing and primer extension to generate the modified *ryjA*. The reverse primer carried a 38 nt substitution that functioned as an efficient synthetic transcription terminator and lacked complementarity to the 3' end of SoxR. In silico modulation of modified RyjA revealed that the *ryjAS3* formed the stem loop structure similar to the wild type RyjA (**Fig. 3.1.21 b**).

The 151 bp amplicon by overlap extension PCR was cloned in pNEB206AT and the recombinant pRyjAS3 was confirmed by digesting with PstI and SacI and further by sequencing (**Fig 3.1.20**; sequencing results included in Appendix).

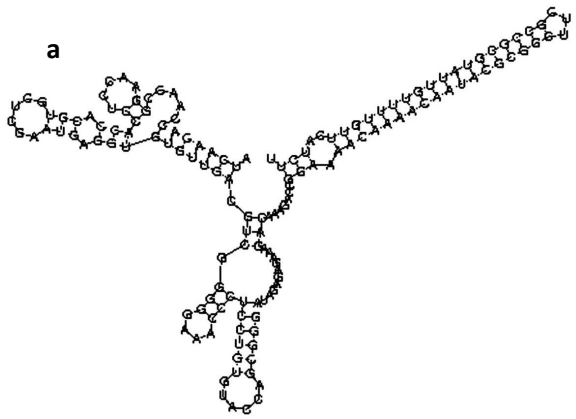


Fig. 3.1.20: Nucleotide sequence and secondary structure of *ryjA* substitution mutant. (a). The substituted nucleotide sequence (boxed).

(b). The predicted secondary structure of substituted *ryjA*, *ryjAS3*.

b

RyjA substitution sequence (151 bp):

ATCAACACCAACCGGAACCTCCACCACGTGCTCGAATGAGGTGTGTTGACGTCGG
GGGAAACCCTCCTGTGTACCAGCGGGATAGAGAGAAAGACAAAGACCGGAAAAC
AAAACAATACGCGGCTTCGCCGCGTATTGTTTGTTCATCTT

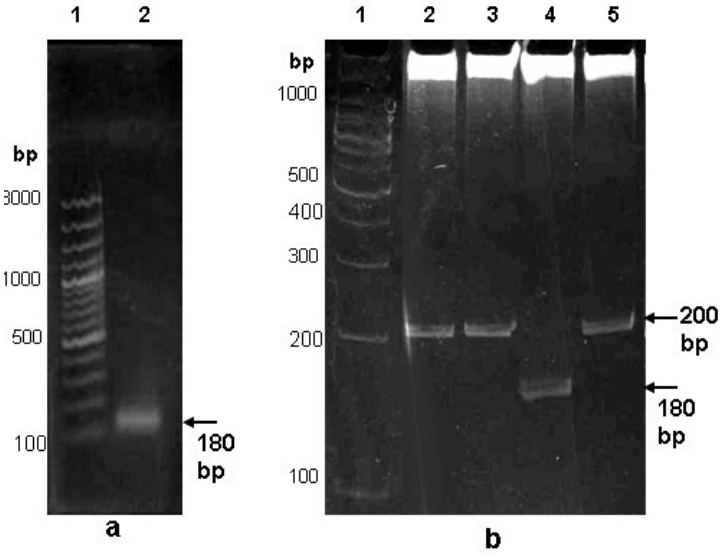


Fig. 3.1.21. Construction of RyjA substitution mutant, pRyjAS3:

(a). Amplification of RyjAS3 by appropriate primers. Lanes: 1, 100 bp MW marker; 2, RyjAS3 amplicon (180 bp). **(b). Confirmation of pRyjAS3 by restriction digestion of recombinants and running the samples on 6% PAGE.** Lanes: 1, 100 bp MW marker; 2-5, recombinants digested with PstI and SacI where lanes 2,3 and 5 show release of native *ryjA* (200 bp) and Lane 4 indicates an insert release of 180 bp.

3.1.6.5. Analysis of effects of RyjA modifications

Plasmids carrying RyjA with independent deletion of different stems enabled the assessment of role of individual stem domain essential for RyjA activity. The effect of these deletions on SoxR expression was evaluated by SoxR-dependent induction of *soxS′-′lacZ* fusion in strain BW1157 (**Fig.3.1.22**). As discussed in section 3.1.5.1., the over expression of wild type *ryjA* resulted in down regulation of *soxS′-′lacZ* expression whereas modified *ryjA* with stem 3 deletion did not exhibit any such

down-regulation and was comparable to that of vector control. The RyjA substitution mutant pRyjAS3 with non complementary 3' end to SoxR sequence did not influence the *soxR* expression as reflected by *soxS′-′lacZ* expression similar to that in the vector control.

The induction of the stem 2 deletion derivative (pRyjAΔ2) resulted in a moderate reduction of *soxS* expression, between that of pRyjA and pRyjAΔ3 suggesting the possible involvement of stem2 in indirect regulation of *soxR*, probably via interacting with other targets of RyjA predicted by TargetRNA program (Section 3.1.3.3). Nevertheless the down regulation of *soxS* expression by multicopy RyjA and its comparable expression in vector control or when stem 3 was deleted or substituted, indicate a specific involvement of this stem in mediating the negative regulatory effect.

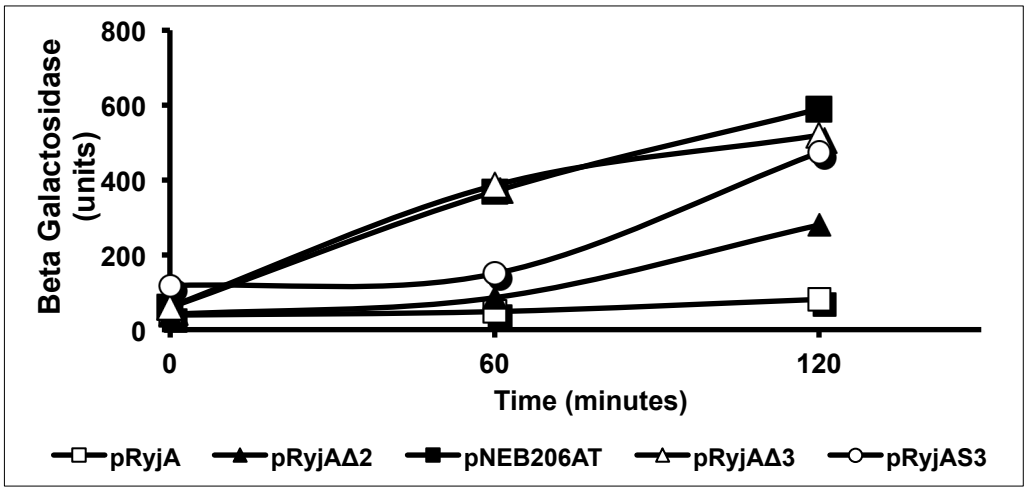


Fig.3.1.22: *soxS'-'lacZ* expression under overexpression of RyjA (wild type) and RyjA deletion mutants: Overnight cultures of BW1157 (pRyjA), BW1157 (pRyjAΔ2), BW1157 (pRyjAΔ3), BW1157 (pRyjAS3) and BW1157 (pNEB206AT) were inoculated in LB (1% v/v). The expression of *ryjA* deletion mutant was induced by IPTG (20 µg ml^{-1}) and the oxidative stress by paraquat (50µM) at an optical density of ~ 0.4 at 600. The data represented is mean of three experiments.

3.1.7. Northern analyses of RyjA expression in pRyjA

The levels of RyjA transcripts and its potential interaction with other RNAs were studied by Northern blot of total RNAs from different strains, using *ryjA* and *soxR* as probes. Total RNAs were prepared from strains overproducing RyjA from IPTG induced *lac* promoter in pRyjA and isogenic control strains bearing the vector, all grown till an OD_{600} of 0.4. A parallel set of pRyjA was additionally stressed with PQ.

RyjA and SoxR transcripts were identified by their size in separate hybridizations with corresponding probes. Expression of *ryjA* increased in a time dependent manner after IPTG induction and this increase negatively correlated with the levels SoxR (**Fig. 3.1.23**). The decrease of SoxR transcripts in the presence of pRyjA was similar to that observed for *soxS'-'lacZ* expression described above.

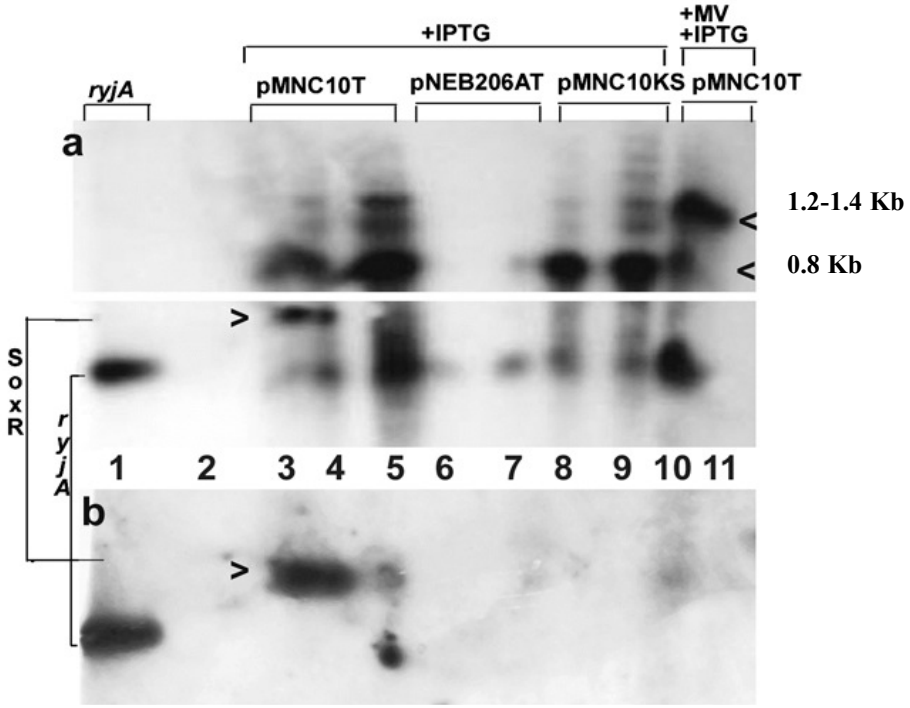


Fig 3.1.23: Northern detection of RyjA and interacting transcripts: Total RNA (30 μg) from BW1157 (pRyjA) and (pNEB206AT), grown till an OD_{600} of 0.4 and induced by IPTG, with or without PQ, were separated on 6-8% urea-poly acrylamide gels and hybridized using digoxygenin labelled *ryjA* and *soxR* probes. Full length *ryjA* and *soxR* amplicons were run in parallel as controls. **(a)** Lane 1: RyjA amplified (150 nt). Lanes 3-5: BW1157 (pRyjA) under IPTG induction 0.5, 4 and 6hrs respectively. RyjA (140 nt) and *soxR* (~470 nt) transcripts are indicated. Lanes 6-7: BW1157 (pNEB206AT) bearing only chromosomal copy of *ryjA* used as control, treated by IPTG + PQ. Lanes 8-9: pMNC10KS by IPTG induction, 4 & 6 hrs respectively. Lanes 10-11: BW1157 (pRyjA) induced by IPTG and PQ at 0.5 hrs & 4 hrs respectively. The levels of two unknown transcripts (~0.8 kb and 1.2 kb) are influenced by oxidative stress. **(b)** The same blot re-hybridized with SoxR probe A full length amplified SoxR was used for comparison.

Since the *ryjA* probe was double stranded, both strands could have hybridized to the other transcripts on the blot. Whether the hybridisation was due to interactions by RyjA or its complementary strand to mRNAs was assessed by bioinformatic analysis (20-24 bases). However no significant hit was found. It is possible that since the hybridization conditions were less stringent it allowed detection of other transcripts. But due to weak signals they were ignored. The transcripts observed in the blot are probably different conformers of *soxR-ryjA* transcripts formed due to mild denaturing conditions and would have migrated slowly.

That these RNAs are not due to transcriptional read through from *ryjA* to *lacZ* in pRyjA was confirmed by the absence of any hybridization signal to the *lacZ* probe.

3.1.8. Northern analyses of RyjA expression in various RyjA mutants

The expression of RyjA transcript in *ryjA* over expression, deletion, substitution and disruption mutant strains was studied by Northern blot of total RNAs using *ryjA* as a probe. Total RNAs were prepared from strain BW1157 overproducing RyjA or derivatives of RyjA from IPTG induced pRyjA, pRyjAΔ2, pRyjAΔ3, pRyjAS3 and the *E. coli ryjA* disruption mutant, RyjADM. The isogenic strain bearing the vector pNEB206AT served as the control. A parallel set of all these strains was additionally stressed with PQ.

RyjA transcripts were identified by high stringency northern hybridization with the corresponding probe and the molecular weight of transcripts was confirmed by RNA marker. The RNA load in all the wells is indicated by the 23S and 16S rRNA bands in the corresponding well of the agarose gel (**Fig. 3.1.24**).

The RyjA transcripts were detected only for strains expressing these from inducible *lac* promoter in multicopy plasmid. Strains bearing single copy *ryjA* or disrupted *ryjA* did not yield positive signals (**Fig. 3.1.24**, Lane 3-4, Lane 5-6), although weak positive bands were seen in the control strain in the blot shown in Fig. 3.1.23.

Multicopies of full length RyjA transcript were observed in *ryjA* over expressing strain pRyjA while a lower molecular weight *ryjA* transcript was expressed from the *ryjA* stem 2 deletion plasmid in pRyjAΔ2 (**Fig. 3.1.24** , Lane 1 & 7). A decrease in the RyjA levels was observed under PQ (oxidative stress) for both, pRyjA and pRyjAΔ2. The pRyjAΔ3 which lacked the RyjA 3' end sequences yielded multiple bands of higher molecular weight. The absence of appropriate transcription termination sequences in the cloned r*yjAΔ3* resulted in extended transcripts (**Fig. 3.1.24** , Lane 9-10). The expression of RyjAS3 was also verified in northern blot and was increasing expressed with IPTG induction from 2 to 4 hrs (**Fig. 3.1.25**, Lanes 1 & 3).

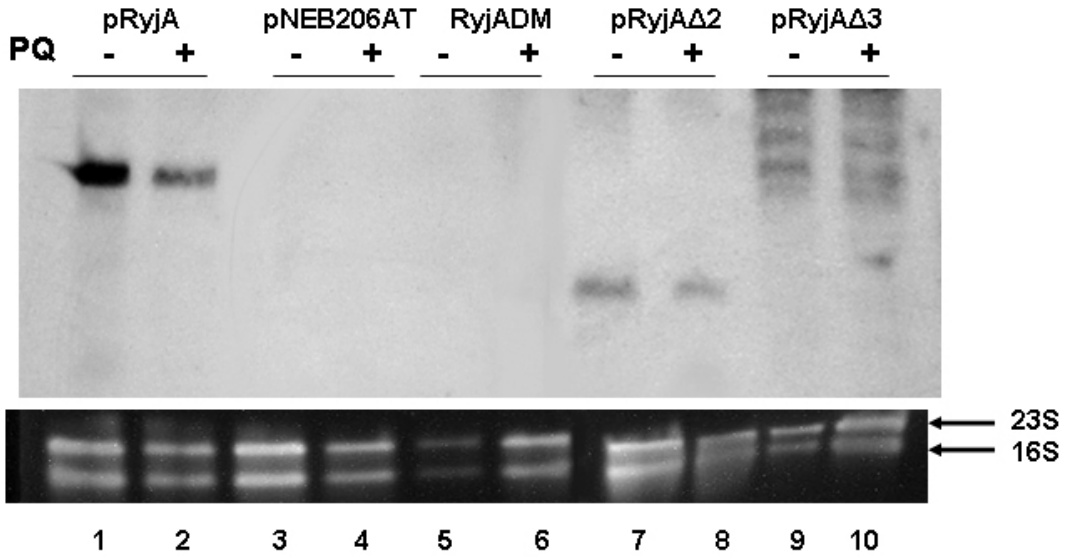


Fig. 3.1.24: (a) Northern detection of RyjA in various RyjA derivatives. Total RNA was extracted from IPTG induced (20 µg/ml at OD_{600} of 0.4) and untreated or PQ-treated (100 µM for 60 min at OD_{600} of 0.8) cultures. Following cultures were used, BW1157 (pRyjA), BW1157 (pNEB206AT), *E. coli* RyjADM, BW1157 (pRyjAΔ2) and BW1157 (pRyjAΔ3). 30 µg of total RNA was separated on 8% urea-polyacrylamide gels and hybridized using *ryjA* probe. -, absence of PQ; +, presence of PQ. A replicate 1% agarose gel was run and stained with ethidium bromide (EtBr), revealing the 16S and 23S rRNA, which serves as loading control.

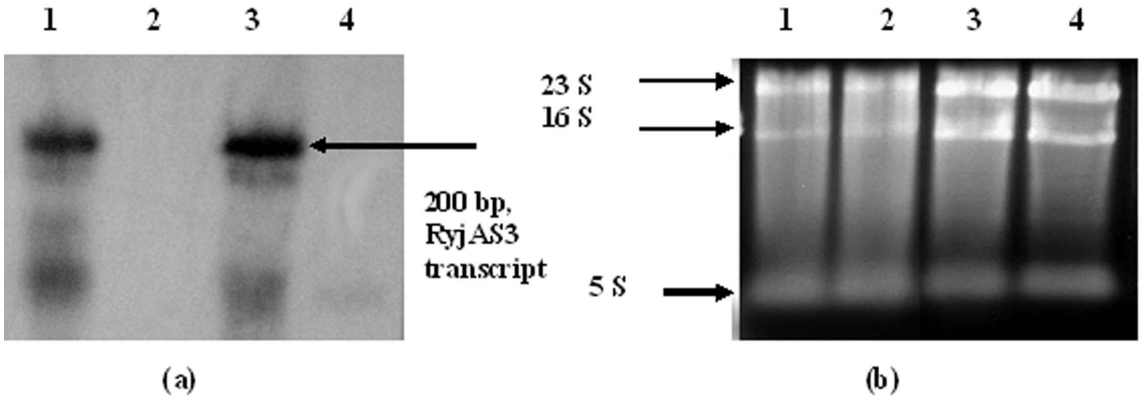


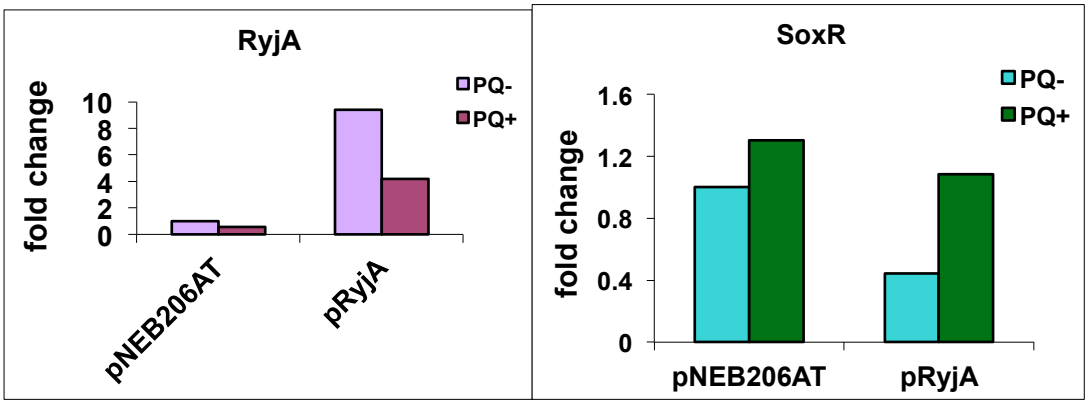
Fig. 3.1.25: (a) Northern blot for pRyjAS3 and pNEB206AT & (b) RNA loading control (~ 2.5 µg) for northern blotting. Lanes: 1 & 3, pRyjAS3 (2hrs and 4hrs after IPTG induction); 2 & 4, pNEB206AT (2hrs and 4hrs after IPTG induction).

3.1.9. Analysis of transcripts within the *soxRS* regulon by real time qPCR

The mRNA levels of *soxR*, *soxS*, *sodA* (Mn superoxide dismutase) and *nfo* (endonuclease IV) under the influence of multicopies of RyjA, or RyjA derivatives (pRyjAΔ3) and (pRyjAS3), and disrupted *ryjA* were measured by quantitative real time PCR. The BW1157 (*soxS'-'lacZ*) strain bearing the above plasmids and the

ryjA disruption mutant were induced with IPTG and PQ for RNA isolation and cDNA synthesis (section 2.3.3). The control strain containing the empty vector BW1157 (pNEB206AT) was used as the reference strain. The 16S rRNA served as an endogenous control for the qRT-PCR analysis

The decreased levels of RyjA under PQ stress as detected by northern blot (Section 3.1.8) were further confirmed by qRT-PCR in the RyjA overexpressing and the vector bearing strain. The PQ untreated pRyjA cells indicated a 9 fold increase in RyjA transcripts than the single chromosomal copy in the reference strain. These levels decreased by 5 fold upon paraquat stress but were still 8 fold more than the corresponding paraquat induced control (**Fig. 3.1.26 a).** However when compared within vector control, the expression of RyjA decreased by 0.5 fold upon induction by PQ. Parallely the induction of *soxR* transcripts was observed under paraquat treatment; yet the levels of *soxR* transcripts were less in pRyjA as compared to the corresponding control indicating a down-regulation by RyjA multicopies (**Fig. 3.1.26 b).**



(a) **(b)**

Fig. 3.1.26: Analysis of RyjA and SoxR levels under the absence and presence of oxidative stress: (a) Fold change in *ryjA* transcript levels in PQ treated and untreated pNEB206AT and pRyjA. (b) Fold change in *soxR* transcript levels in PQ treated and untreated pNEB206AT and pRyjA.

In addition to *soxR* other representative genes of SoxRS regulon- soxS, sodA and nfo were also down-regulated under the influence of multicopies of RyjA **(Fig. 3.1.27**). Conversely the downregulation of all these genes was abrogated and further increased in the RyjADM, the disruption mutant (**Fig. 3.1.27**).

Strains bearing RyjA modified derivatives, pRyjAΔ3 and pRyjAS3 lacking the sequences complementary to the 3' of SoxR had increased levels of *soxR* and other transcripts of the *soxRS* regulon implicating that these modified derivatives either interfere with normal RyjA function or influence by an excess of stem1/ stem 2 loops. The levels of *nfo* were conspicuously high in both these *ryjA* derivatives implicating that perhaps the excess copies of loop 1 and 2 had a positive effect on *nfo* levels (**Fig. 3.1.27**).

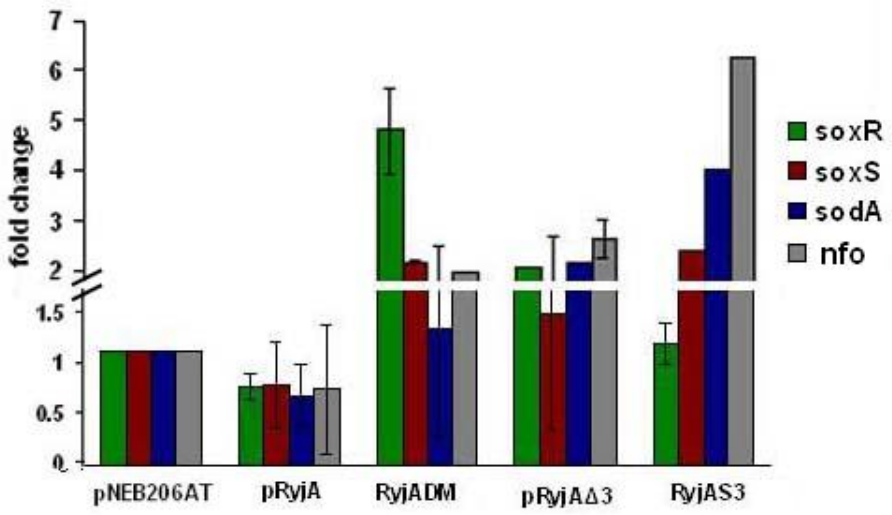


Fig. 3.1.27: Analysis of mRNA levels under overexpression of RyjA (wild type), RyjA derivatives and RyjA disruption. Regulation of *soxR* and the consequent effect on the expression of *soxS*, *sodA* and *nfo* in various RyjA derivatives relative to the control strain.

The *ryjA* was disrupted in *E. coli* DY330 strain containing λ recombinase. Since the all the *ryjA* multicopy constructs were analysed in *E. coli* BW1157 host background, any effect of genotypic differences on expression of SoxRS regulon were tested. Almost similar transcript levels of *soxS*, *nfo* and *ygbN* were noted for *E. coli* BW1157 and *E. coli* DY330 (**Fig. 3.1.28**).

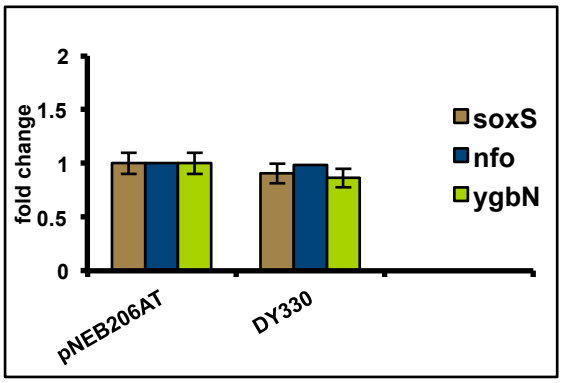


Fig. 3.1.28: Analysis of fold change in transcript levels of *soxS*, *nfo* and *ygbN* in BW1157 (pNEB206AT) and *E. coli* DY330 . Results shown are representative experiments performed at least twice.

of

3.1.10. Discussion

3.1.10.1 RyjA alters SoxR expression

The exposure of *Escherichia coli* to superoxide anion stress generated by redox-cycling drugs such as paraquat (methyl viologen), menadione (MD) and phenazine methosulfate (PMS) activates the SoxR transcription factor by oxidation of its [2Fe-2S] cluster (Gu and Imlay, 2011). The redox-cycling drugs are compounds that are released by both plants and bacteria as devices that penetrate into the cell interior of the competitors and inhibit the growth. The redox-cycling drugs abstract single electrons from the reduced flavins or metal centers of redox enzymes and transfer the electron to oxygen, generating superoxide (Inbaraj and Chignell, 2004).

The oxidized SoxR activates the transcription of SoxS which then stimulates the expression of several genes. A few known SoxS-activated genes include *sodA* (Mn super oxide dismutase), *nfo* (endonuclease IV), *zwf* (glucose-6-phosphate dehydrogenase) and *fumC* (fumarase C). A time series microarray identified additional co-regulated SoxRS dependent and independent genes, either up or down regulated, few transcriptional regulators, putative transcription factors and six small RNAs to be a part of paraquat response model (Blanchard *et al*., 2007).

The RyjA sRNA has an 18 bp complete complementarity with the transcription termination region of the *soxR* and is positioned convergently to SoxR This study shows that the ectopic expression of RyjA sRNA down-regulates the expression of the chromosomally encoded SoxR, probably by pairing with complementary sequences overlapping the transcription terminator region of the *soxR* mRNA.

The slow growth rate and increased sensitivity to ampicillin imparted by pRyjA can be justified by the report of Greenberg et al., 1990 wherein SoxR positively regulated the expression of Mar regulator of multiple antibiotic resistance. The regulation of SoxS by SoxR is well established. The 3' overlap of RyjA with SoxR and the reduction in *soxS′-′lacZ* expression under multicopy RyjA support the interpretation that RyjA negatively influences *soxR* transcript levels. The reduction in *soxR, soxS, sodA and nfo* transcripts in real time PCR further supported this. The amount of *soxS* mRNA and therefore the transcriptional activation of its target genes is strongly dependent on the presence of active Fe-SoxR (Hidalgo and Demple, 1994). Taken

together all the observations in this work suggested a net negative effect of RyjA on expression of SoxR.

The decrease in *soxR* expression could not have been by mere coincidental base pairing interactions of *ryjA* due to high copies; since the abrogation of the downregulation of SoxR was noticed when chromosomal *ryjA* was disrupted.

The expression of RyjA from native promoter as well as *lac* promoter was reduced under paraquat stress. The sigma factor RpoS that regulates the expression of RyjA is negatively regulated by sRNA OxyS under oxidative stress (Altuvia *et al*., 1997). Therefore the induction of OxyS under oxidative stress might explain low levels of *ryjA* transcripts thus permitting increased expression of SoxR.

The reduced RyjA under PQ stress manifested to an increased level by 4 hrs as observed in Northern blots. It is conceivable that after few hours, the metabolic perturbation induced by PQ could be tapered to normal physiology bringing an increase in *ryjA* when SoxR is inessential.

The increased levels of RyjA in the absence of oxidative stress might help to decrease the basal level of SoxR protein thereby preventing its wasteful expression. This fine tuning is required for reducing the response time and quick transition of cell physiology when oxidative stress is induced. The fine tuning of gene expression by sRNA regulators in response to external stimuli is a common phenomenon (Shimoni *et al*., 2007). The fine tuning of CsgD synthesis, the stationary phase-induced biofilm regulator, when switching from the motile-planktonic to the adhesive-sedentary state by differential and temporal expression of three sRNAs RprA, GcvB and McaS was reported recently (Jørgensen *et al*., 2012).

The expression of *soxR* gene is also subjected to auto regulation as the binding of SoxR to the single site that mediates *soxS* activation also represses *soxR* transcription (Hidalgo *et al*., 1998). This would allow constant level of SoxR to be maintained under oxidative stress. The regulation of SoxR expression by RyjA sRNA provides an additional layer of regulation to avoid any unnecessary translation of SoxR and probably represents an example of feed-forward loop that comprise a repressor and a sRNA (both regulating the same target). The feed forward loop is a three-gene pattern and composed of two input transcription factors, one of which regulates the other, both jointly regulating a target gene (Mangan *et al*., 2006). In the present study it is proposed that the RyjA is the second regulator of the expression of SoxR autoregulator. In few cases the transcripts that are produced despite the transcription repression are taken care of by the sRNA. The feed-forward loops suit conditions where it

is crucial to completely abolish expression of a gene, as in the case of *ompF* under high osmolarity (Shimoni *et al*., 2007).

The observation of increased expression or increased transcripts of SoxS and associated genes in pRyjAΔ3 and pRyjAS3 suggest that RyjA mediates regulation of SoxR through the stem 3. It was expected that the excess copies of stem 3 modification would have no net effect on the function of chromosomal *ryjA* as opposed to dwnregulation of SoxRS regulon by pRyjA. However, it was intriguing to observe that the stem 3 deletion or substitution by bases which did not base pair with the *soxR* terminator caused a large increase in the *soxR, soxS, sodA and nfo* transcript levels than the strain containing the wild type RyjA. Such an observation could be explained by an assumption that the RyjA modified derivatives inhibit the function of wild type RyjA by competitive binding to the target molecule or titrating out the protein required for its regulatory mechanism.

The terminator stem loop of RyjA needs to be opened to promote the *soxR-ryjA* complex formation. There has to be some *in vivo* factor, most likely a protein or RNase cutting within the loop of stem 3 of RyjA. The sequence of stem 3 region of RyjA is highly conserved in Enterobacteriaceae family and energetically unstable making it a potential region to initiate interaction with target mRNA. It is known that for efficient sRNA-mRNA interaction the region of interaction initiation must be located in highly accessible regions in both interaction partners or unstructured conserved sRNA region (Richter and Backofen, 2012). The interaction between RyjA and *soxR* may also be influenced by the secondary structure of the *soxR* transcript which has not been characterized.

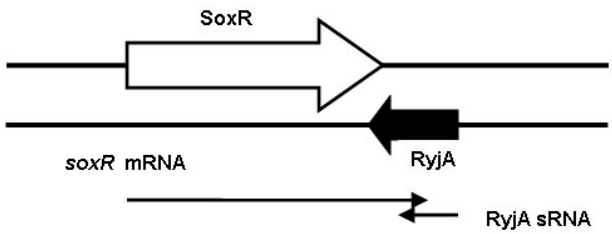


Fig. 3.1.29: Probable map of overlap between terminator stem loop region of RyjA sRNA and *soxR* mRNA. The block arrows represent the genes on the double stranded DNA.

For the majority of known bacterial antisense sRNA the terminator stem-loop does not participate in the sRNA:mRNA interaction and most of them interact with their target genes around the targets' translation start sites and ribosome binding sites (Tjaden *et al*., 2006). However only a few sRNAs are known where the terminator stem-loop participates in the sRNA:mRNA interaction. The most well studied being the *cis*-encoded GadY sRNA of *E. coli* and *trans* encoded SR1 sRNA of *B.subtilis*. The gene arrangement couples the GadY small RNA to *gadX* mRNA in *E. coli* and mediates the base pairing at the 3' end of both the transcripts (Opdyke *et al*., 2004). GadY positively regulates the expression of *gadX* evident by accumulation of *gadX* transcript upon over expression of *gadY*.

In case of the SR1 sRNA (205 nt) of *B.subtilis*, the 78 nt in the terminator stem-loop region (spannig from 109 nt to 186 nt of the sRNA) efficiently forms complex with its target *ahrC* mRNA and regulate the expression. The *ahrC* mRNA is the transcriptional activator of the *rocABC* and *rocDEF* arginine catabolic operons (Heidrich *et al*., 2007).

The genomic overlap of *ryjA* and *soxR* is very suggestive of the base pairing interactions between the two. Such double stranded mRNA pairs are very easy targets for degradation by several nucleases (Morita *et al*., 2005) and therefore explain the negative regulation on expression of SoxR by RyjA. This mechanism would be opposite to the one proposed for GadY that also uses 3' base pairing for the stabilization of its target GadX.

Very little is known about the 3' UTRs of bacterial mRNA and the type of regulatory signals they contain. A lot has been learned about the regulation at the 3' UTR of eukaryotic transcripts. Several microRNAs in eukaryotic organisms have been shown to form base pairs with the 3' UTR of their target mRNAs (Nelson *et al*., 2003). Any mutations that occur within the pairing region will automatically create a compensatory change on the opposite strand, with no net loss in complementarity between the small RNA and its mRNA target.

3.1.10.2 RyjA might regulate multiple targets

A few *E. coli* sRNAs such as RyhB, OxyS etc. are known to regulate more than one target under a specific stress or growth condition (Masse *et al*., 2002; Altuvia *et al*., 1997). Among these, the OxyS also functions as a pleiotropic regulator during

oxidative stress (Zhang *et al*., 1998). Therefore the possibility of RyjA regulating multiple targets which are encoded at separate locations of the chromosome is not ruled out. The TargetRNA program, which predicts targets for small RNAs, identified four mRNAs as targets for RyjA sequence which are YgeZ, YhfK, YgbN and YhhJ. Although sequence similarity suggests that the YgbN is a proton-driven metabolite transporter belonging to the Gnt family of gluconate transporters, the cloned *ygbN* gene did not confer gluconate transport (Keseler *et al*., 2005). While YhhJ is a putative inner membrane protein with five predicted transmembrane domains belonging to the ABC superfamily of transporters (Rudd, 2000), YgeZ, also known as HyuA codes for hydantoin-utilizing enzyme and has similarity to allantoinase enzymes but the function is unknown (Rudd, 2000). The target YhfK is a conserved integral membrane protein and belongs to YccS/YhfK family. Overexpression of *yhfK* from a plasmid confers resistance to the toxic chemical, bromoacetate (Keseler *et al*., 2005).

The hybridization of *ryjA* probe to multiple RNAs in northern blot and the stem 3 substitutions used in this studies mediating an unusual increase in several SoxRS transcripts are probably indications of involvement of more than one stem of RyjA in target regulation. The possibility of RyjA regulating multiple targets requires further work.

3.1.10.3 RyjA shows complete complementarity to a hypothetical protein Q1R3K6_ECOUT

While the 3' end of *ryjA* is complementary to the 3' end of *soxR*, its 5' 120 bp is complementary to the sequence of a putative uncharacterized protein Q1R3K6_ECOUT (88 amino acid) of *E. coli* (UT189/ UPEC) (accession no. Q1R3K6). This protein coding gene also exists in *E. coli* K12 genome but has not been annotated. The FASTA for Q1R3K6_ECOUT protein of Uniprot KB protein database also suggested the presence of this putative uncharacterized protein in various strains of *E.coli*. No functional domains for protein Q1R3K6_ECOUT were indicated on analysis by Prosite.

3.1.10.4 Antisense *cis*-sRNA in *E.coli*

RyjA appears to be the member of a class of regulatory RNAs encoded on the DNA strand opposite their target RNAs. The RNAs which are transcribed from the

antisense strand of a known transcriptional unit are called as antisense RNAs (asRNA). Naturally occurring antisense RNAs were discovered more than 40 years ago in bacteriophage and plasmids (Spiegelman et al., 1972; Tomizawa *et al*., 1981; Stougaard *et al*., 1981). A recent transcriptome analysis confirmed the widespread antisense transcription in *E. coli* by identifying about 1000 different asRNAs (Dornenburg *et al*., 2010). Bacterial asRNAs are diverse and can be classified based on their location as 5'-overlapping (divergent, head to head), 3'-overlapping (convergent, tail to tail), or internally located asRNAs (George and Hess, 2011). As *ryjA* is convergently positioned to *soxR* the RyjA sRNA belongs to the 3'-overlapping class of asRNAs.

The asRNAs have been reported to: alter the target RNA stability (*E. coli* GadY sRNA), modulate translation (*E. coli* SymE sRNA), transcription termination (*Shigella flexneri* RnaG asRNA) and transcriptional interference (*Clostridium acetobutylicum* asRNA) (George and Hess, 2011).

The initial searches in the database for the possibility of targets exhibiting 3' end similar to that of SoxR for assessing the pleiotropic effect of RyjA did not yield any significant output. However the recently updated database of annotated genes of *E. coli* in 2012 (www.ncbi.nlm.nih.gov/genomes/static/pipeline/html) revealed a few predicted targets putatively belonging to MFS, the major facilitator superfamily transporter having similar sequence. This could mean that the altered physiology under the influence of multicopies of RyjA could be perhaps compounded by the effect on these targets.

The present study carried out in *E. coli* suggested that the RyjA is a *cis*-encoded sRNA that fine tunes by 3' base pairing the expression of SoxR, the key regulator involved in the adaptation of the cell to the oxidative stress physiology.

3.2.Functional characterization of *IsrC*

3.2.1. IsrC the proposed positive regulator of Antigen 43

A bioinformatics approach to search for DNA regions within *E. coli* genome that contain a σ70 promoter within a short distance of a *rho*-independent terminator identified IsrC sRNA among several other small RNAs (Chen *et al*., 2002).

IsrC sRNA (204 nt) initially named as IS102, located at 44.6 minutes on *E. coli* genome is flanked by *yeeP* and *flu* gene on either side and forms a part of CP4-44, the putative prophage remnant (Hershberg *et al*., 2003). The *isrC* gene is positioned upstream of *flu* gene and in sense orientation (**Fig. 3.2.1**). The *flu* gene codes for Antigen 43 (Ag43) while the *yeeP* gene codes for putative GTP binding protein.

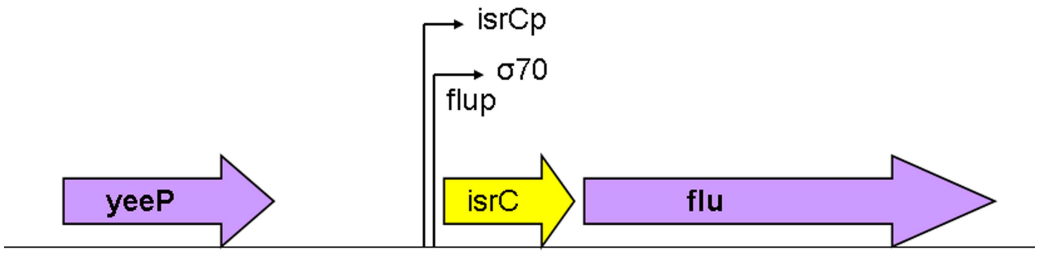


Fig. 3.2.1: Schematic representation of *isrC* genomic location: The *isrC* gene is located in between the *yeeP* and *flu* gene. The sequence of IsrC overlaps with the regulatory region of the *flu* gene. *isrCp* and *flup* are the promoters for *isrC* and *flu* respectively.

The *isrC* sRNA sequence when subjected to *Sfold* (Ding *et al*., 2004) indicated four stem loop structures (**Fig. 3.2.2**). These stem loop structures may work independently or in conjunction to regulate one or many target transcripts.

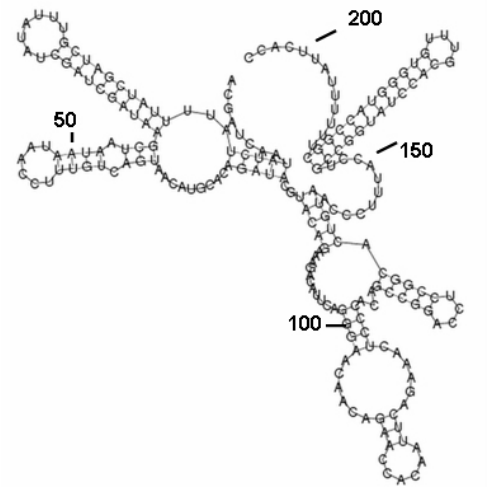


Fig. 3.2.2: Predicted secondary structure of IsrC sRNA by *Sfold.* The number represents the nucleotide in the *isrC* sequence**.**

3.2.2. The cryptic prophage CP4-44

The well-studied *E. coli* K-12 contain nine cryptic prophage elements, which comprise 3.6% of its genome. The CP4-44 is a defective prophage with seven genes including the sRNA gene *isrC* and seven pseudogenes- *yoeA*', *yoeG*', *yeeW*', *yoeF*', *yoeH*', *yoeD*', and *yeeP*' (Blattner *et al*., 1997). Although identified as a putative prophage-derived element, CP4-44 lacks signature capsid or assembly genes to confirm its origins. This contiguous prophage interval and its length include two insertion sequences which are not prophage genes, IS5H and IS2F. IS5H interrupts the *yeeH'* and *yoeG'* pseudogenes and IS2F interrupts the *yoeA'* gene (**Fig. 3.2.3**). The CP4-44 deletion in *E. coli* K12 greatly reduced the biofilm formation and ability to aggregate (Wang *et al*., 2010).

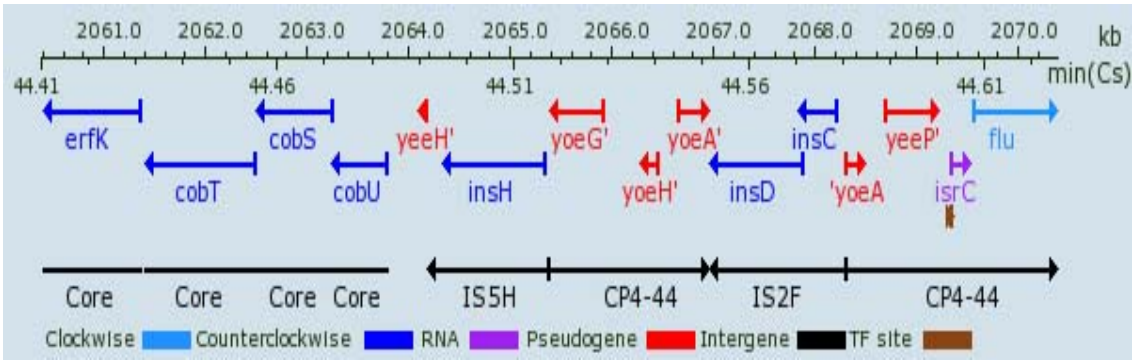


Fig. 3.2.3: Genomic location of cryptic prophage CP4-44: The genomic localization of cryptic prophage CP4-44 on the *E. coli* K12 genome indicating the position of the small RNA *isrC*, pseudogenes, *flu* gene and the insertion sequences (ww.ecogene.org).

3.2.3. Antigen 43

Antigen 43 (Ag43) is encoded by the *flu* gene (for fluffy morphology) situated at the boundary of CP4-44 43 min on the *E. coli* K-12 chromosome. The *flu* gene is a part of the cryptic prophage CP4-44 present either as a single copy in *E. coli* K-12 or as multiple alleles in several *E. coli* strains including enteropathogenic and uropathogenic strains.

Ag43 is a phase variable (with frequency of 10^{-3} per cell) outer membrane, auto transporter surface protein and is present in ~50,000 copies. Ag43 possesses the typical auto transporter protein domains: an N-terminal signal peptide; an N-proximal

passenger domain or α43, that is secreted and a C-terminal β-barrel domain that forms an integral outer membrane protein (β43) (**Fig. 3.2.4**). Expression of Ag43 promotes frizzy colony morphology and bacterial cell-to-cell aggregation (autoaggregation) which can be visualized macroscopically as flocculation and settling of cells in static liquid suspensions (van der Woude and Henderson, 2008). Ag 43 is a multifunctional protein which promotes biofilm formation on various surfaces as well as bacterial binding to some human cells and enhances bacterial tolerance to bactericidal agents (Fexby *et al*., 2007).

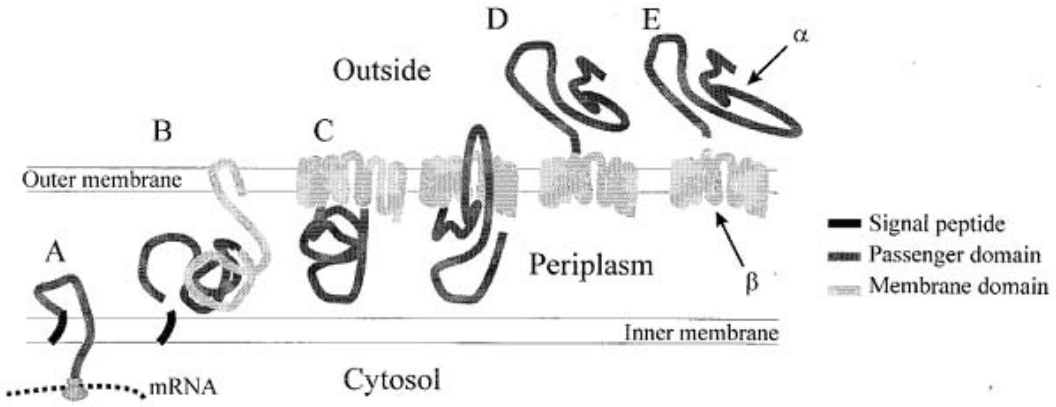


Fig. 3.2.4: Model of the biogenesis and processing of the Antigen 43 autotransporter protein. The signal peptide (black) enables the autotransporter to reach the periplasm (A). Subsequently, the β-membrane domain (light gray) starts inserting into the outer membrane (B) where it forms a pore (C) through which the α-passenger domain (dark gray) is translocated to the cell exterior (D). Once the α domain has reached the cell surface, it is processed by autocatalysis but remains associated with the cell through interaction with the β domain (E) (Adapted from Kjaergaard *et. al.*, 2000).

Ag43 increases the pathogenicity of *E. coli* enteropathogenic and uropathogenic strains by initial recognition and attachment to host tissue surfaces and enhancing biofilm forming ability (Hagan *et al*. 2007; Ulett *et al*., 2007). Kjaergaard *et al*., 2000 reported the expression of Ag43 in other gram negative organisms such as *Pseudomonas fluorescence* and *Klebsiella pneumoniae* and observed the changes in the colony morphology.

3.2.4. Regulation of Ag43 expression

The main regulatory feature of Ag43 expression is its phase variation where the cells in a clonal population either express the gene (On phase) or they do not (Off phase) (Diderichsen, 1980; Henderson *et al*., 1997 (a); Henderson and Owen, 1999). Phase

variation is a common feature mainly of surface proteins of commensal and pathogenic bacteria and is heritable yet reversible (Henderson *et al*., 1997(b); Ulett *et al*., 2006). The On to Off and Off to On phase variation occur not only in K-12 but also in other wild type strains (Roche *et al*., 2001).

Phase variation of Ag43 requires specific sequence elements in the regulatory region and two proteins: OxyR and Dam methylase (van der Woude, 2006). OxyR represses *agn43* transcription by binding to the regulatory region of *agn43*, which overlaps the –10 of the promoter sequence, resulting in the Off phase. The On phase is obtained by methylation of the three GATC sequences by Dam in the regulatory region, which abrogates the binding of OxyR at the GATC-containing regulatory region. Therefore the methylation of the *agn43* GATC sequences and OxyR binding are mutually exclusive, and the expression state is determined by the outcome of the competition between these two proteins for the *agn43* regulatory region (**Fig.3.2.5**). DNA replication must occur for new, unmethylated sequences and thus is required for the On to Off switch. It may also be required for the Off to On switch to displace OxyR and allow Dam access to sites.

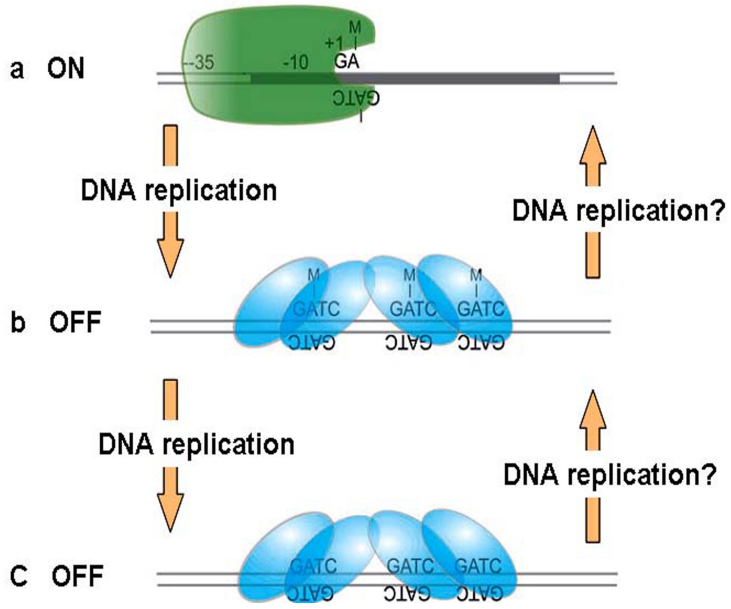


Fig. 3.2.5: Schematic depiction of regulatory control of *agn43* transcription by OxyR and Dam. Shown are both strands of DNA with the Dam target sequences GATC. Methylation is depicted with an M, RNA polymerase is shown in green, OxyR as a dimer of dimers is in blue, the promoter is labelled –10 and –35, and the transcription start site is +1. (*a*) The On phase with fully methylated sequences that block OxyR binding. (*b*) An intermediate Off phase with hemimethylated DNA and bound OxyR. (*c*) The Off phase with unmethylated DNA and OxyR bound with higher affinity than in panel *b*. (Adapted from van der Woude and Henderson., 2008).

3.2.5. Screening for sRNA targets

3.2.5.1. Target prediction program

The web based TargetRNA (Tajden *et al*., 2006) is a program that calculates optimal hybridization scores for sRNA-target RNA hybrids and gives a ranking list of candidate mRNAs. The program is based on characteristic features of the known antisense-target interactions found in *E.coli*. Target mRNAs were searched for IsrC, based on Target RNA program that predicted five targets, among which ycgX, b0362 & yghG are hypothetical proteins and other two - sseB and yfcU - are serine enhanced sensitivity protein and a putative outer membrane protein respectively (**Table 3.2.1**).

Table 3.2.1. List of predicted targets of IsrC sRNA by TargetRNA.

Target gene product	**sRNA:Target interaction**	***E. coli* align-ment score**	**P value**
YcgX: Hypothetical protein	**sRNA 35 GAUCGAUAAGCUAAUAAUAACCU-UUGUCAGU 65** **|||| || || |||||||||| |||| |||** **mRNA 7 CUAGGUAGUCC--UAUUAUUGGAGAACAAUCA -23**	-74	0.0024
SseB: Enhanced serine sensitivity	**sRNA 102 ACAACAGAACCACAAUUCA 120** **||| |||||||||||||** **mRNA 13 UGUAUUCUUGGUGUUAAGU -6**	-73	0.0029
b0362: Hypothetical protein	**sRNA 35 GAUCG-AUAAGCUAAUAAUA 53** **|||	|||||||||||||||** **mRNA 8 CUACCGUAUUCGAUUAUUAU -12**	-72	0.0034
YghG: Hypothetical protein	**sRNA 167 GGUAUCCACGUUUGUGGGUACCGGCUUUUUUAUUC 201** **||:|| ||||||| |:	||| |||||||||** **mRNA 5 CCGUAUGUGCAAAAAU--A-GGC---AAAAAUAAG -24** ***E. coli* alignment score: -67** **P value** 0.00779048		
YfcU: Putative outer membrane protein	**sRNA 10 AUCUAUUUUAUCGAUCGUU-UAUAUCGAUCGAUAAGCUAAU-AAUAA 54** **||	||||||||| |:| || || :||| ||||	|| |||||** **mRNA 17 UAAACAAAAUAGCU-GUACCAUUUA-UUAGAGAUUCCAAUAAUUAUU -28** ***E. coli* alignment score**: -66 **P value** 0.00916565		

3.2.5.2. BLAST search

The target genes for the uncharacterized *E. coli* sRNAs, were identified by BLAST search. The BLASTX search for *isrC* targets indicated 92% identity and 40nt match with Ag43 protein suggesting the high possibility of the IsrC regulating the expression of *agn43*.

Table 3.2.2. BLASTX result for IsrC

sRNA (bp)	Similarity Match(amino acid)	Alignment Display	Identities
IS102(204)	Ag43 (1091)	Target 1--------42 sRNA 77--------196	92%

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3.2.6. Ag43 selected as the target for further analysis

Among the potential targets identified for IsrC sRNA (Ag43, SseB, YcgX, b0362, YghG and YcfU), the regulation of Ag43 expression was analyzed as the other targets indicated uncharacterized proteins with putative functions. A possible up-regulation or down-regulation of Ag43 was studied by autoaggregation and expression of Ag43 mRNA and protein in strains expressing altered levels of IsrC.

3.2.7. Analysis of the effect of multicopies of IsrC on Ag43 expression and the consequent physiology

3.2.7.1. Construction of IsrC and anti IsrC over expressing plasmid

In order to construct the IsrC over expressing plasmid (pIsrC), the *isrC* gene alongwith 30 bp upstream and 40 bp downstream was amplified by primers IsrCFP and IsrCRP and cloned into multicopy plasmid pBluescript SK(-) (pBSSK$^-$) vector under the strong and inducible *lac* promoter. The amplicon was digested with BamHI and HindIII (**Fig. 3.2.6. a**) and ligated to the digested linearized pBluescript KS(-) vector (using similar restriction enzymes as used for amplicon). The recombinants obtained were confirmed by insert release where an insert of 320 bp was obtained upon digestion with BamHI and HindIII restriction enzymes (**Fig. 3.2.6. b**). Further they were confirmed by sequencing (Appendix). The over expression of IsrC was later verified by northern blot as mentioned in 3.2.4.2.

For antisense expression, the *isrC* was cloned in the plasmid pBluescript KS(+) such that the template strand of *isrC* was transcribed from the *lac* promoter in the vector, upon IPTG induction. The recombinants were confirmed by restriction digestion and insert release of ~ 350 bp (**Fig. 3.2.6. c**). Subsequently the IsrC over expressing plasmid, pIsrC and the control plasmid pBSSK$^-$ were transformed into *E. coli* MC1061.

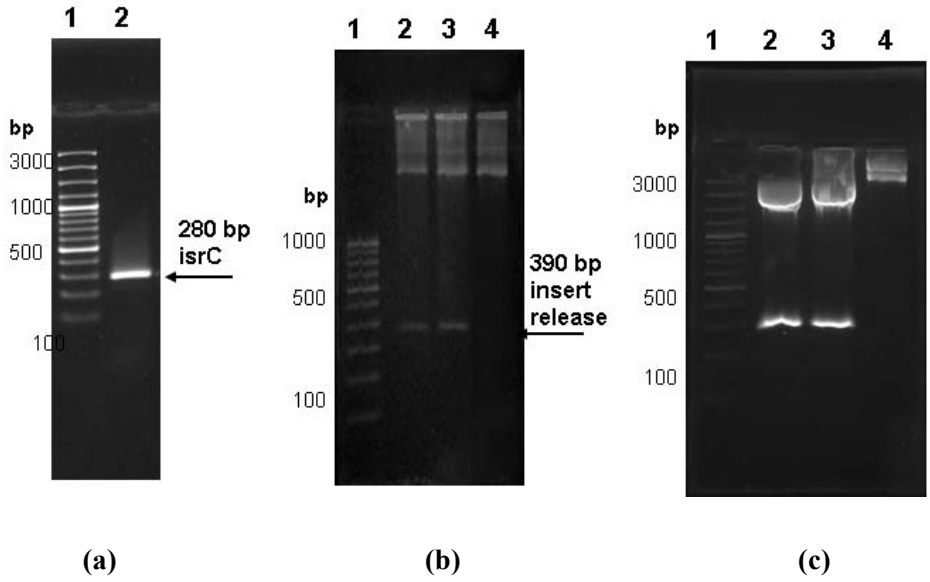


Fig. 3.2.6: Construction of *isrC* and anti *isrC* overexpressing strains: **(a) Amplification of *isrC***: The *isrC* was amplified from *E. coli* genomic DNA using high fidelity X-Taq DNA polymerase. Lanes: 1, 100 bp ladder; 2, ~ 280 bp *isrC* amplicon. **(b) Confirmation of pAntiIsrC**: Lanes: 1, 100 bp ladder; 2, & 3, recombinants digested with BamHI and HindIII indicating an insert release of 320 bp; 4, Undigested pIsrCOvex. **(c) Confirmation of pIsrCOvex**: Lanes: 1, 100 bp ladder; 2, & 3, recombinants digested with BamHI and HindIII; 4, Undigested pAntiIsrC.

3.2.7.2. Northern Blot analysis

The *isrC* transcripts were assessed by northern blot. RNA was isolated from *E. coli* MC1061 separately carrying pIsrC, pAntiIsrC and pBSSK$^-$ at an optical density of ~ 0.8 OD at 600 nm and were induced with IPTG. 20 µg RNA from a 1-2 hour induced culture was separated on 1 % formaldehyde agarose gel and studied by northern blot using *isrC* dsDNA probe. The dsDNA probe could detect both the sense and antisense *isrC* transcripts. The blot probed with *isrC* indicated the presence of *isrC* transcript of 240 bp in MC1061 (pIsrC) strain. Moreover one unknown transcript of higher molecular weight (500 bp) was also observed. Whether this is due to transcription initiation or termination on additional sites at *isrC* locus on the chromosome is not clear. Since RNA was isolated from the late log phase culture

no transcript was detected in control strain probably as *isrC* is expressed in late stationary phase. However in case of strain over expressing antisense *isrC*, the northern blot resulted in higher molecular weight transcripts (1000 bp, 800 bp and 370 bp) indicating the lack of transcription termination sequences at the 3' end of antisense strand (**Fig. 3.2.7**). The higher molecular transcripts suggest the possibility of transcription termination within the *lacZ* fragment downstream. Therefore the pAntiIsrC, the anti *isrC* over expressing strain was not used for further studies.

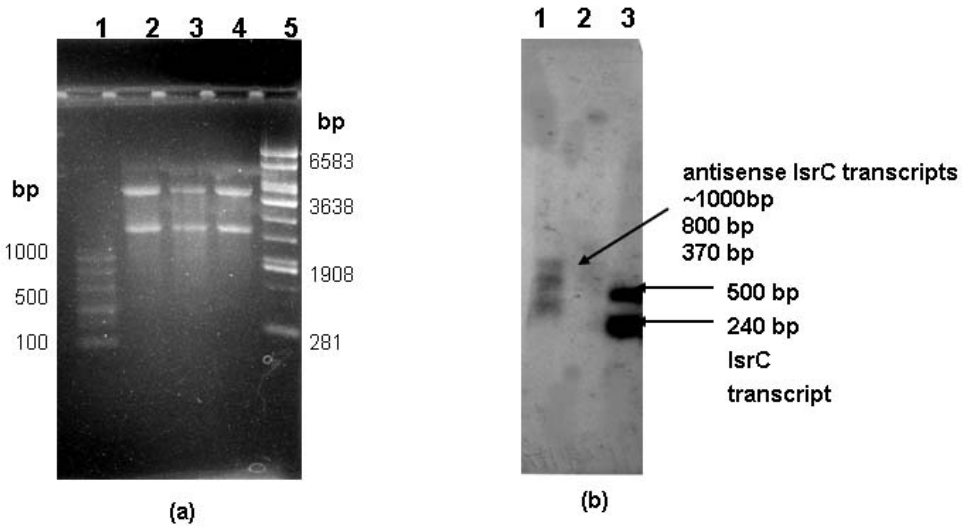


Fig. 3.2.7: Northern blot analysis of pIsrC, pAntiIsrC and pBSSK⁻ : (a) 1% formaldehyde agarose gel. Lanes: 1, RNA marker (100 bp-1 kb); 2-4, RNA from pAntiIsrC, pBSSK⁻ and pIsrC; 5, RNA marker (1 kb- 6.6 kb). **(b)** Northern blot. Lanes: 1, pAntiIsrC; 2, pBSSK⁻ ; 3, pIsrC

3.2.7.3. Analysis of the expression of Ag43 by real time qPCR

The transcript levels of Ag43 were measured in *isrC* over expressing strain, MC1061 pIsrC and control strain with MC1061 (pBSSK⁻) by quantitative RT-PCR using Step One Real time PCR systems. Cultures were induced with IPTG (20 µg/ml) and total RNA was isolated at around 1.0 O.D at 600 nm. The isogenic strain MC1061 (pBSSK⁻) served as the reference strain while 16S rRNA was taken as the endogenous control. The expression of *agn43* under the influence of multicopies of IsrC was up-regulated by 1 .6 fold (**Fig. 3.2.8**). This relative increase in Ag43 expression indicates the positive modulation by *isrC* sRNA. The increased Ag43 expression was further correlated by immunodetection of Ag43 protein and autoaggregation assay.

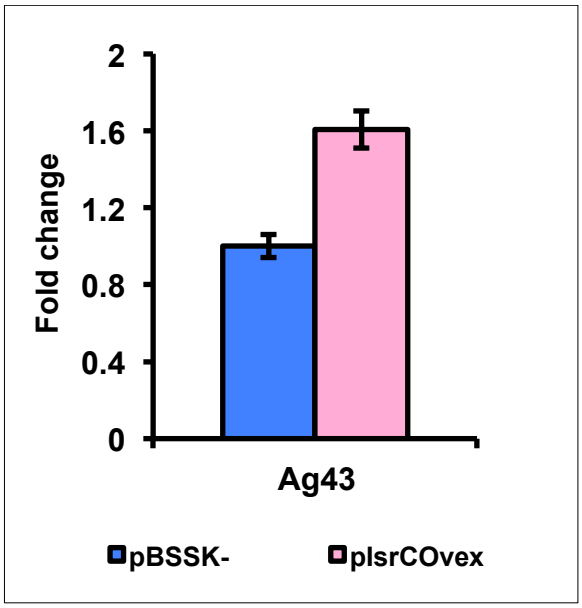


Fig. 3.2.8: Analysis of expression of Antigen 43 by qRT-PCR in MC1061pIsrC and pBSSK$^-$ strains: RNA was isolated at 1 OD_{600} and 2 µg was used to make the first strand cDNA. The cDNA was diluted five times and 1µl was used as a template along with 2X SYBR green master mix and 900 nM of each Ag43 primers, AgnFP and AgnRP. The fold change in expression of Ag43 was calculated by $^{\Delta\Delta}$Ct method and 16S rRNA served as endogenous control.

3.2.7.4. Comparison of Ag43 protein expression in MC1061 pIsrC, MC1061 (pBSSK$^-$) and UPEC *E.coli*

It has been reported that α subunit of Ag43 is responsible for Ag43-Ag43 self recognition (Klemm *et al*., 2003). Expression of Ag43 altered by IsrC sRNA was measured at the protein level by immunodetection (Indirect ELISA) using antiserum against the Ag43 α subunit and was compared in uropathogenic *E. coli* strain, *E. coli* MC1061pIsrC and *E. coli* MC1061 (pBSSK$^-$). Strains grown till 24 hours were used for ELISA. The protein was prepared as discussed in section 2.12.1 and estimated by Folin Lowry method. 40 µg of diluted protein (antigen) was coated in wells of a microtitre plate and incubated at 4°C overnight. The ELISA was performed as discussed in section (2.12.2.). For all the three primary antibody dilutions i.e. 15000, 10000 and 5000 and the cultures grown till 24 hrs, a two fold increase in the ELISA titre of Ag43 antibody was observed in *isrC* over expressing culture compared to the control and the uropathogenic *E. coli*. The increase in the titre of Ag43 antibody in the *isrC* over expressing culture reflected increased expression of Ag43 (**Fig 3.2.9**). Comparative high levels of Ag43 protein in the presence of IsrC multicopies further

corroborates the results of RT-PCR and confirms the increased Ag43 expression and therefore it's positive modulation by IsrC sRNA.

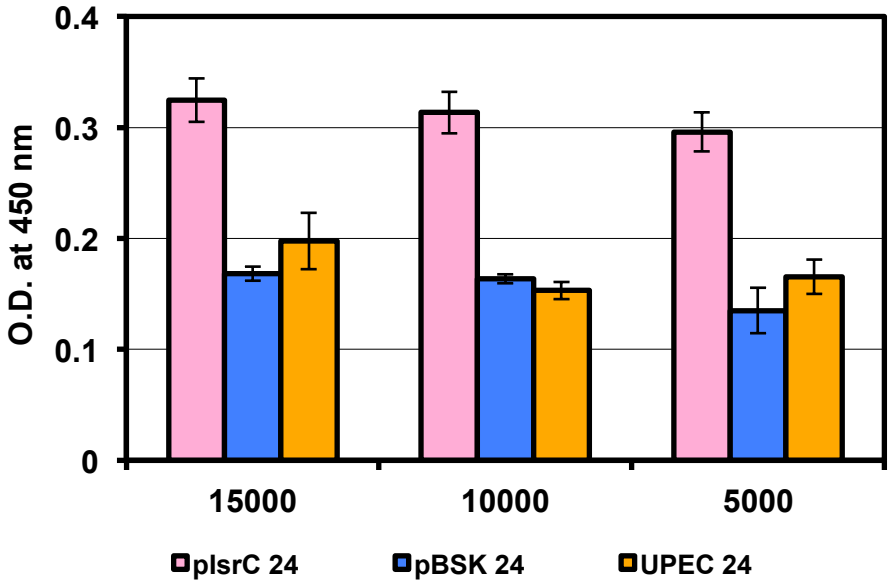


Fig. 3.2.9: Detection of Ag43 by indirect ELISA: The α subunit of Ag43 protein was detected by indirect ELISA for uropathogenic *E.coli*, *E. coli* MC1061 pIsrC and *E. coli* MC1061(pBSSK$^-$) strains. The cultures were grown till 24 hrs and the α subunit of Ag43 was isolated. Different primary antibody dilutions i.e. 1:5000, 1:10000, 1:15000 were used.

3.2.7.5. Effect of multicopies of IsrC on autoaggregation

Some *E. coli* strains express very high number of Ag43 surface protein, which promotes bacterial cell to cell aggregation (autoaggregation) seen as characteristic flocculation and settling of cells from static liquid suspensions. Functionally bacterial autoaggregation enhances biofilm formation and cell adhesion, both of which are closely related with bacterial virulence. Hence autoaggregation confers the organism an ability to resist various host defences e.g. complement attack and phagocytosis which are essential for virulence mechanism.

The increased expression of Antigen43 under the presence of multicopies of IsrC was expected to lead to abundant Ag43 autoaggregator at the bacterial surface and therefore increased autoaggregation. The differential expression of Ag43 was assessed by autoaggregation assay in *E. coli* MC1061 pIsrC and *E. coli* MC1061 (pBSSK$^-$). A hospital isolate of uropathogenic *E. coli* strain (UPEC) was used as a positive control to study the autoaggregation phenotype since uropathogenic strains

have been reported to produce a strong aggregation phenotype and promote significant biofilm growth (Ulett *et al*., 2007). The UPEC strain CFT073 has been reported to contain two copies of the Ag43-encoding *flu* gene located on pathogenicity islands. Expression of Ag43 in these strains has been reported to result in rapid flocculation and settling of cells from standing overnight cultures (Ulett *et al*., 2007).

The autoaggregation for MC1061 pIsrC, MC1061 (pBSSK$^-$) and UPEC was monitored by bacterial settling over time at 0°C. The cultures were grown from 12 hours to 48 hours in nutrient broth containing meat extract 37°C and 180 rpm. As Ag43 is involved in pathogenicity, the use of nutrient broth was expected to facilitate its expression and increase the autoaggregation. The schematic representation of autoaggregation assay is depicted in **Fig. 3.2.10.**

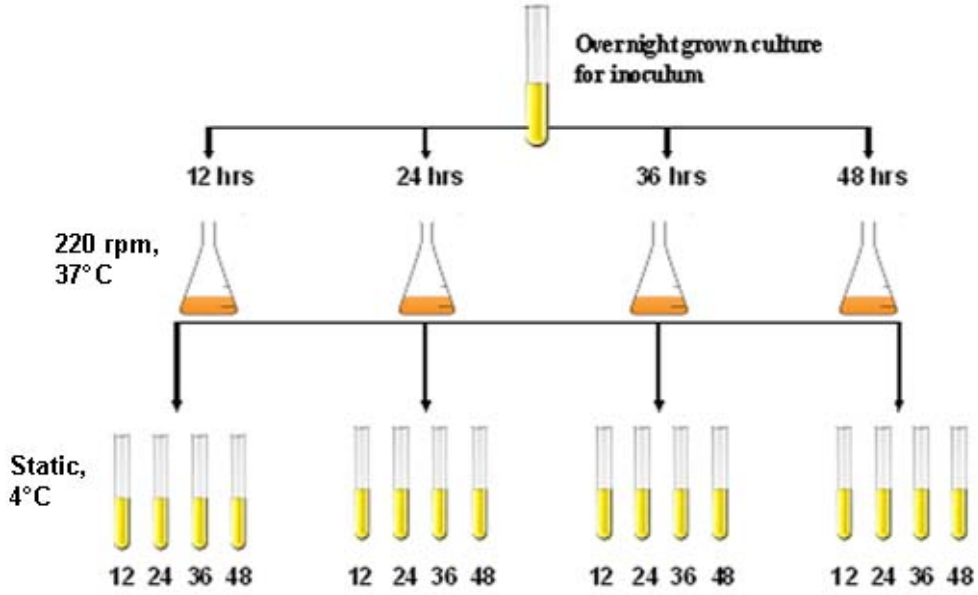


Fig. 3.2.10. Schematic representation of the autoaggregation assay. 1% of overnight inoculum was used to inoculate LB in four different flasks. From the cultures grown at 37°C and 180 rpm for 12 hrs, 24 hrs and 48 hrs, 5 ml of culture was dispensed in four test tubes which were shaken vigorously for 10 seconds and allowed to stand still for 12 hrs, 24 hrs and 48 hrs in cold room at 0°C. 100µl of culture was withdrawn very carefully from the top of each test tube and mixed with 900µl of 0.9% NaCl. The OD was measured at 600 nm.

All the standing liquid cultures indicated noticeable difference in rate of settling of cells in the first 12 hours of the autoaggregation assay where the slow, gradual and differential sedimentation of cells to the bottom of the tube was observed. Later the

rate of settling of cells was not marked (**Fig 3.2.11**). When compared within the cultures, UPEC and *isrC* over expressing strain grown till 12 hrs exhibited increased autoaggregation than the vector containing strain (**Fig 3.2.11.a**). For the cultures grown till 24 hrs the uropathogenic strain exhibited significant autoaggregation. In contrast, the difference in the settling rate of cells was less pronounced for the cultures MC1061 pIsrC and MC1061 (pBSSK$^-$) (**Fig 3.2.11. b**). After 48 hours of growth all the cultures had almost similar profiles for settling of cells (**Fig 3.2.11. c**). The increased Ag43 expression in pIsrC did not result in significant enhancement in autoaggregation but was intermediate between the UPEC and control strains. This is probably because several other surface adhesins other than Ag43 also cause clumping and subsequent aggregation of the cells.

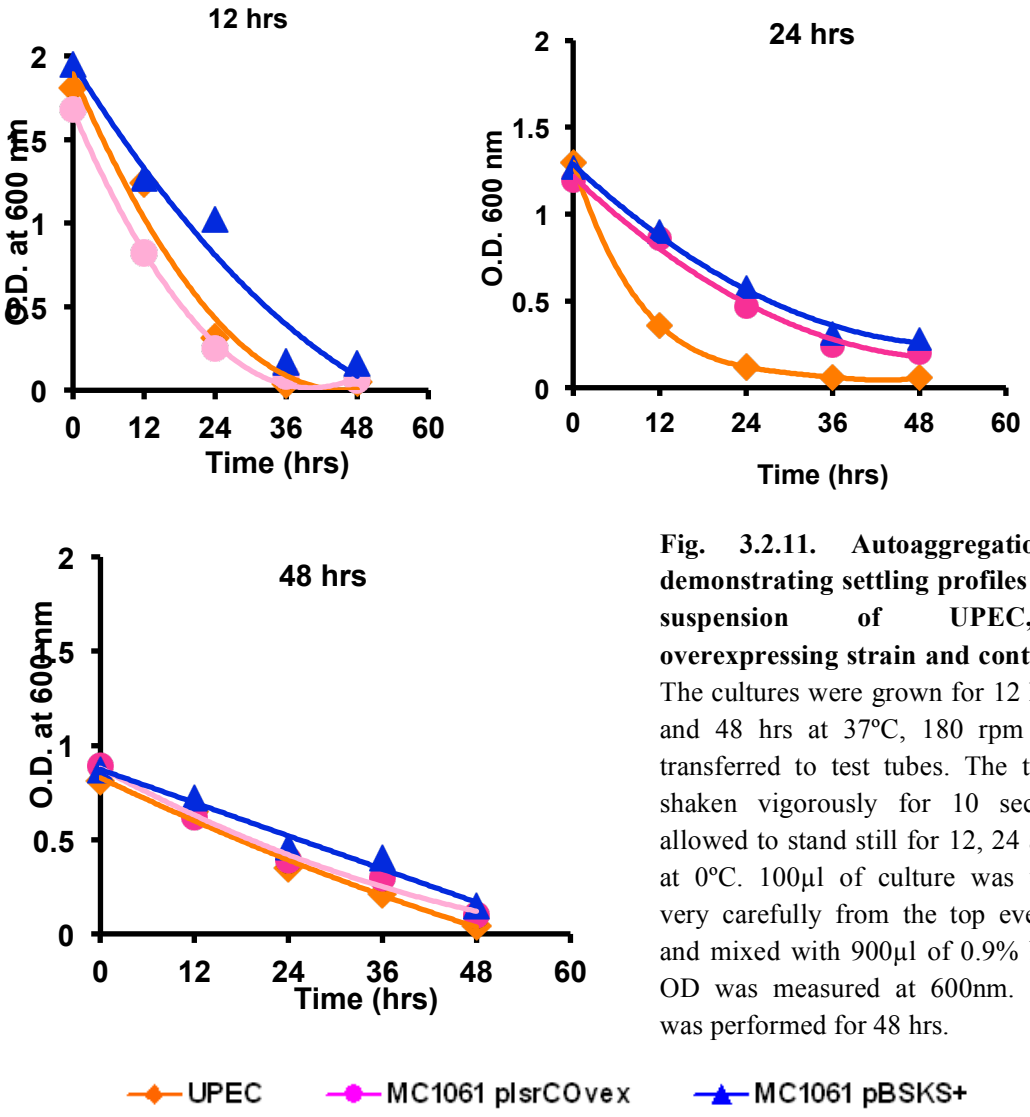


Fig. 3.2.11. Autoaggregation assay demonstrating settling profiles for liquid suspension of UPEC, *isrC* overexpressing strain and control strain. The cultures were grown for 12 hrs, 24 hrs and 48 hrs at 37ºC, 180 rpm and were transferred to test tubes. The tubes were shaken vigorously for 10 seconds and allowed to stand still for 12, 24 and 48 hrs at 0ºC. 100µl of culture was withdrawn very carefully from the top every 12 hrs and mixed with 900µl of 0.9% NaCl. The OD was measured at 600nm. The assay was performed for 48 hrs.

3.2.7.6. Macrophage: Bacteria interactions

An additional proposed role for Ag43 when expressed from a high copy plasmid is that expression promotes uptake and survival in polymorphonuclear neutrophils (PMNs) and increased virulence (Fexby et al., 2007). When the THP1 macrophage cells were incubated with *isrC* over expressing strain in a ratio of 1:200 for 90 minutes and analyzed by FACS it was observed that MC1061 (pIsrC) had an increased uptake and survival rate. The flow cytometry indicated higher number of fluorescently labelled and non labelled *isrC* over expressing bacteria within the macrophage (approximately 30% more) in comparison to the control culture. However the above observation inconsistent probably because the slight modulation of Antigen 43 expression did not facilitate marked uptake and survival in macrophages.

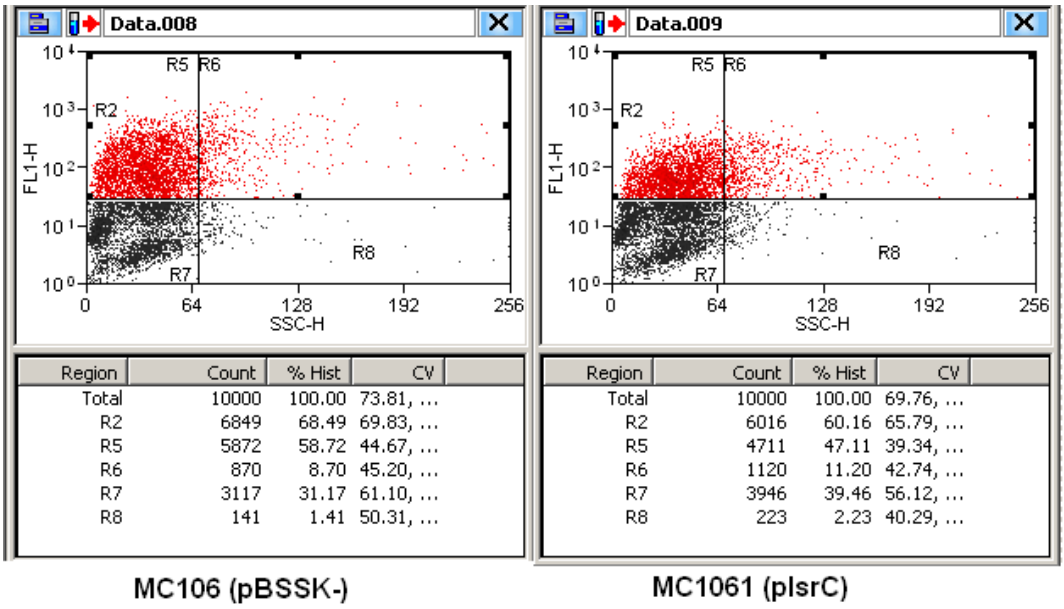


Fig. 3.2.12. A representative of the FACS analysis. The cultures pIsrC and pBSSK- were incubated with THP1 macrophages in a ratio of 1:200 for 90 min and analysed by FACS. The quandrants R6 and R8 represent the total number of bacteria phagocytosed by macrophages.

3.2.8. Discussion

The phase variation of Ag43 expression is regulated at the transcriptional level by OxyR mediated repression (OFF phase) at the three equally spaced GATC sites in the regulatory region of *flu*. The Dam methylation at these sequences abrogates the binding of the OxyR and derepresses the expression of Agn43 (van der Woude and Henderson, 2008). The expression of Ag43 is also regulated indirectly by RfaH protein. The RfaH protein is a transcriptional antiterminator and regulates many operons including lipopolysaccharide (LPS) biosynthesis operon which physically shields Ag43 preventing its exposure to the cell surface. A mutation in *rfaH* depleted the lipopolysaccharide (LPS) consequently unmasking and increasing the Ag43 protein quantity that lead to a strong adhesion and biofilm formation (Beloin *et al*., 2006). Additionally two small RNAs, OmrA and OmrB which when expressed at high levels negatively regulated the expression of several genes encoding multiple outer membrane proteins including *flu*. Although these observations were inconsistent, the authors still suggested that OmrA and OmrB could repress *flu* expression (Guillier and Gottesman, 2006). In the present work, IsrC is proposed as a probable additional positive regulator of Ag43 expression.

3.2.8.1 Sequence characteristics of IsrC sRNA

The IsrC sRNA houses the regulatory region of the *flu* gene spanning from 3 to 38 nucleotides that includes the three OxyR binding GATC sites. The sequence of *isrC* was analysed in the present study. The promoter prediction by *BPROM* indicated two σ70 promoter sequences upstream of *isrC*. The promoter for *isrC* is located 30 nucleotides upstream of the *flu* promoter (*fluP*) that is 3 base pair upstream of *isrC* transcription initiation site. The *rho*-independent terminator of *isrC* identified by *Findterm* (section 2.12) extends from 164th nucleotide to 201 nucleotides. Downstream the *isrC* gene (20 nucleotides) is the translation initiation site of the *flu* (**Fig.3.2.13**). These analyses suggest an overlap of IsrC gene with promoter of *flu*.

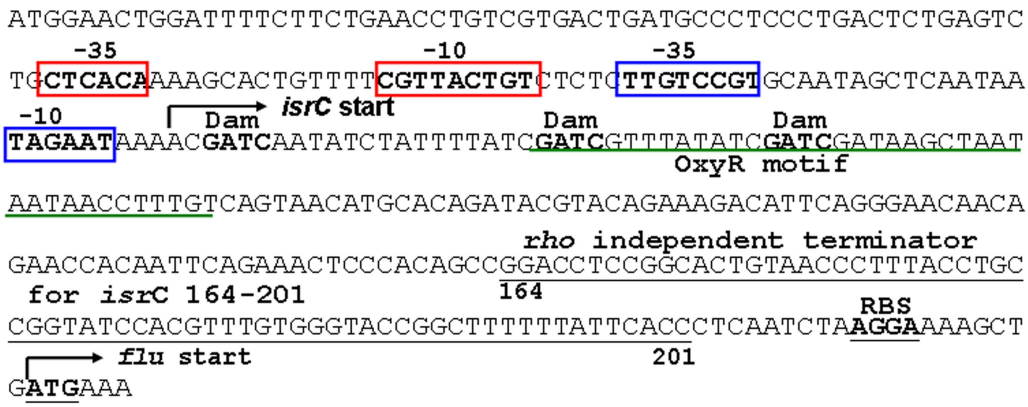


Fig 3.2.13: The sequence features of i*srC*: The promoter region for *flu* (blue box) and *isrC* (red box) gene, the transcription initiation site and *rho* independent terminator of *isrC*, Dam methylation sites, suggested OxyR-binding site, ribosomal binding site (RBS) and translation initiation site for *flu* is indicated.

The *isrC* expression is under the control of PhoP/PhoQ two component system and is repressed in the presence of PhoP (Raghavan *et al*., 2011). The PhoP/PhoQ signalling system responds to low magnesium and presence of cationic antimicrobial peptides and regulates genes important for growth under these conditions, as well as additional genes important for virulence in many gram negative pathogens (Lippa and Goulian, 2009). However the expression of IsrC is independent of Mg^{2+} levels suggesting that the PhoP-mediated regulation responds to an additional cue (Raghavan *et al*., 2011).

In the present study the overexpression of IsrC resulted in 1.6-fold increase in *agn43* transcript levels. Our observation for the two fold increase in the antibody titre against the alpha domain of Ag43 in presence of IsrC multicopies in comparison to the control strain and UPEC further corroborated the up-regulation of Ag43 expression.

An increase in expression of Ag43 was anticipated to give higher settling of cells. However both MC1061 pIsrC and UPEC *E. coli* had almost similar settling profiles and only a modest increase than the control. This observation was probably because several proteins have been implicated in rendering autoaggregation phenotype. The deletion of *flu* gene for example did not affect the autoaggregation mediated via the polysaccharide adhesin poly-β-1,6-*N*-acetyl-d-glucosamine (PGA) (Thomason *et al*., 2012). In addition, Ag43 mediated rapid flocculation and settling of cells from standing cultures is observed for *flu* expressing from the multicopy plasmid (Ulett *et*

al., 2006). On the other hand, in the present study the autoaggregation mediated by Ag43 expressed from the single copy of chromosomal *flu* gene is assessed.

The high autoaggregation of the UPEC strain despite a lower ELISA titre of Ag43 than in pIsrC could be justified on the basis of many reports. A serum directed against the alpha-domain of Ag43 from *E. coli* K12 reacted most strongly against the overexpressed K-12 Ag43 alpha-domain subunit than the two variants of UPEC CFT073, Ag43a and Ag43b probably because of the variable sequence of the alpha domain of Ag43 in uropathogenic and commensal *E. coli* strains (Ulett *et al*., 2007).

The alpha subunit of Ag43 is known to be involved in intercellular Ag43-Ag43 binding and therefore aggregation. UPEC *E. coli* have been reported to form biofilm-like structures and possess different *agn43* alleles where one or both the alleles could promote biofilm formation (van der Woude and Henderson, 2008).

Ag43 when expressed from a high copy plasmid results in an increased uptake and survival in polymorphonuclear neutrophils (PMNs) and increased virulence (Fexby *et al*., 2007). When the THP1 macrophage cells were incubated with *isrC* over expressing strain in a ratio of 1:200 for 90 minutes and analyzed by FACS it was observed that MC1061pIsrC had an increased uptake and yet a higher survival rate. The flow cytometry indicated higher number of fluorescently labelled and non labelled *isrC* over expressing bacteria within the macrophage (approximately 30% more) in comparison to the control culture. In the second set of same experiment however no difference was observed in the uptake by macrophages of *isrC* over expressing strain compared to the vector bearing strain. The effect of the positive modulation of Ag43 expression by IsrC was not consistent and did not reflect in enhanced virulence, uptake and survival rate in macrophages probably because such an observation was noted under the influence of multicopies of Ag43. The mild modulation of Ag43 observed in the present study probably did not confer the increased virulence phenotype.

However the exact mechanism by which IsrC modulates the Ag43 expression has not been addressed. It is known that several bacterial sRNAs contribute directly or indirectly to target stabilization or positively influence target expression.

The bioinformatics analysis for *isrC* and *flu* sequence did not yield any efficient complementary base pairing between the two suggesting that the IsrC sRNA probably does not mediate regulation of Ag43 expression through base pairing interactions.

It is also known that mRNAs are not the only targets as a number of sRNAs bind to cellular protein and modulate their activity. Therefore it is proposed that IsrC like other protein binding sRNAs such as the transcriptional regulator 6S sRNA, CsrB or GlmZ could bind to RNA binding proteins and regulate gene expression. In the present study it is proposed that IsrC could bind to the σ^{70}-bound, housekeeping form of RNA polymerase regulate transcription initiation. The *E. coli* RNA polymerase has been reported to bind and react with several sRNAs that are involved in transcriptional regulation (Windbichler *et al*., 2008). The increase in copies of IsrC probably increases transcriptional efficiency of RNA polymerase at p*flu*. The IsrC could also bind to transcripts of OxyR, the regulator of hydrogen peroxide stress and the repressor of Ag43 expression. OxyR activates the production of OxyS sRNA that regulates as many as 20 additional gene products (Altuvia *et al*., 1997). Bioinformatics analysis indicated complementary base pairing between OxyR and IsrC forming an energetically favourable mRNA-sRNA duplex liable to degradation. Thus multicopies of IsrC could downregulate OxyR expression which in turn would derepress Ag43 expression. The proposed mechanism for regulation by IsrC from the present study requires further work.

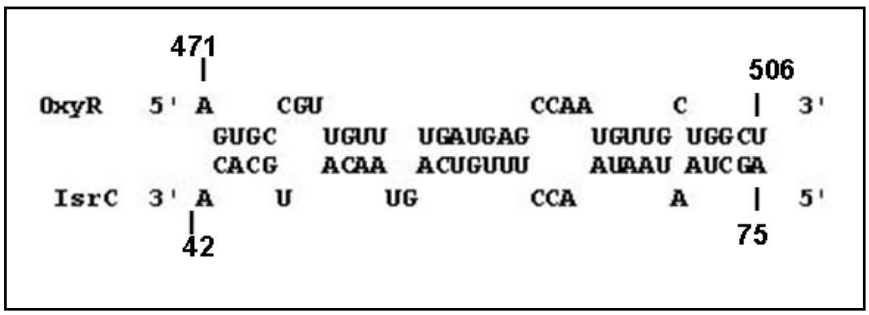


Fig. 3.2.14: Predicted base pairing between IsrC and oxyR mRNA. 5' to 3' direction is indicated for both RNAs.

Summary

Small, non coding, untranslated RNAs are ubiquitous in nature ranging from bacteria to mammals. They serve as regulatory factors for an array of cellular processes including transcription/translational regulation, chromosome replication, RNA processing and modification and even protein degradation and translocation. In *E. coli*, bioinformatics approaches and comprehensive transcript profiling identified ~80 small non coding RNAs (sRNAs) expressed either in stationary phase (DsrA, Spot42, RprA etc) or under environmental stress conditions (RhyB, OxyS, MicF etc).

The BLAST analysis for the sequences of 20 sRNAs with unknown function indicated known protein coding transcripts and hypothetical proteins as targets while the TargetRNA prediction program indicated hypothetical proteins to be targets. This study encompasses the functional characterization of two *E. coli* sRNAs, RyjA (140 bp) and IsrC (204 bp).

RyjA sRNA, expressed in late stationary phase has three stem loops and an 18 bp complete complementarity with the transcription termination region of the *soxR* transcript. The BLAST analysis of *ryjA* showed complementarity only to *soxR* while additional targets including hypothetical proteins with unknown function were indicated by 'TargetRNA'. The SoxR is a redox autoregulator and transcriptional activator of *sox*S, transcriptional regulator that in turn activates the genes of *soxRS* regulon such as *sodA* (Mn superoxide dismutase), *nfo* (endonuclease IV), *zwf* (glucose 6 phosphate dehydrogenase), *fumC* (fumarase C) and several others involved in the oxidative stress management.

For studying the role of RyjA in oxidative stress physiology, strains overexpressing wild type or modified RyjA and disrupted *ryjA* were constructed. Overexpression of RyjA from an IPTG inducible promoter under oxidative stress induced by paraquat was studied which resulted in impaired growth, decreased SoxR dependent expression of *soxS::lacZ* fusion, and reduced levels of *soxR* transcripts. The reduced levels of *soxR* transcripts correlated with reduced levels of *soxS*, *sodA* and *nfo* transcripts. The down regulation of *soxRS* regulon by RyjA was paralleled by deregulation of several other physiological parameters under oxidative stress, such as decreased antibiotic

resistance phenotype and low SOD activity. The chromosomal *ryjA* disrupted strain exhibited increased growth rate and levels of *soxR*, *soxS*, *sodA* and *nfo* transcripts implicating the abrogation of negative effective of RyjA. The transcription of RyjA was reduced by oxidative stress and a concomitant increase in *soxR* and other transcripts was observed. However, the RyjA levels in overexpressing strain were still high under the paraquat stress. The deletion or substitution of SoxR complementary region in stem 3 sequences by terminator sequences lacking complementarity to *soxR* resulted in increased SoxR expression suggesting the possibility of RyjA mediating the regulation by 3' end sequences. The stem 3 substitutions mediated an unusual increase in the several SoxRS transcripts probably indicating the involvement of more than one stem of RyjA in target regulation.

The northern blot of total RNA from oxidative stressed cells probed with *ryjA* revealed additional transcripts changing with induction of oxidative stress indicating the regulation of multiple targets by RyjA. Some of these transcripts had molecular length comparable to those predicted by TargetRNA program suggesting the high possibility of RyjA regulating multiple targets.

The sRNA IsrC (204 nt) is situated at 41.4 minutes on *E. coli* genome. The BLAST analysis indicated that the *isrC* gene overlaps with the 5' regulatory region of the *flu* gene. The *flu* gene codes for Antigen 43 (Ag43), outer membrane and auto transporter surface protein exhibiting a phase variation with a frequency of 10^{-3} per cell.The *isrC* was cloned in both, pBluescript SK- and pBluescript KS+ resulting in the overexpression of sense and antisense IsrC. The northern blot analysis indicated the expected *isrC* transcript length for the sense *isrC* clone and higher molecular weight transcripts for antisense *isrC* clone due transcription termination in the downstream *lacZ*. Overexpression of sense IsrC from the multicopy plasmid resulted in 1.6 fold increase in the expression of Ag43 and a two fold increase in the Ag43 antibody titter. Thus IsrC acts a positive modulator of Ag43 expression. As Ag43 mediates cellular autoaggregation, increased expression of Ag43 was anticipated to accelerate autoaggregation. The autoaggregation under the influence of IsrC was compared to the vector control and a local uropathogenic *E. coli* (UPEC) isolate which is reported to exhibit marked autoaggregation. Equal extent of settling was observed after 48 hrs in all the strains without substantial difference in

autoaggregation. Bacteria-macrophage interaction studies for assessing the consequent influence of increased Ag43 in IsrC overexpressing cells did not yield interpretable change in their uptake by macrophages. The mild modulation of Ag43 probably might not have influenced the bacteria-macrophage interactions. The bioinformatics analysis revealed that there is no complementary base pairing between the IsrC and Ag43 except the overlap at 5' end of Ag43.

Conclusion

The RyjA and IsrC sRNA are *cis*-encoded sRNAs modulating the expression of their targets SoxR and Antigen 43 respectively. The RyjA sRNA in the absence of oxidative stress mediates down-regulation of the expression of redox auto-regulator, SoxR by base pairing of the sequences at the 3' end. The RyjA level is probably maintained in the absence of oxidative stress to prevent the superfluous expression of SoxR. The fine tuning of SoxR expression may be required for reducing the response time and quick transition of cell physiology when oxidative stress is induced. The regulation of SoxR expression by RyjA sRNA provides an additional layer of regulation and probably represents an example of feed-forward loop that comprise a repressor and a sRNA (both regulating the same target). The transcripts that are produced despite the transcription repression are down-regulated by the sRNA. Bioinformatics analysis and experimental evidence indicate involvement of more than one loop and regulation of multiple targets.

The IsrC sRNA appears to be positive modulator of Antigen 43 expression which is already subjected to the regulation by various other regulators such an OxyR, Dam, OmrA, OmrB and RfaH proteins. The regulation by IsrC also appears to be an additional layer of regulation of Antigen 43 expression. The fine control of any gene expression via regulation by multiple regulators could be useful in signal integration when multiple environmental stimuli are present simultaneously.

The regulation of gene expression by sRNAs is more common than previously anticipated. The unique regulatory properties of sRNAs, reduced metabolic cost, the need for additional layers of regulation and faster regulation could explain the employment of sRNAs rather than the protein regulators in responses to certain environmental stress conditions.

Appendix

A.1 Nucleotide sequence

A.1.1. RyjA (wildtype)

>gi|49175990:c4276089-4275930 Escherichia coli K12 substr. MG1655 chromosome complete genome

ATCAACACCAACCGGAACCTCCACCACGTGCTCGAATGAGGTGTGTTGACGTCGGGGGAAAC
CCTCCTGTGTACCAGCGGGATAGAGAGAAAGACAAAGACCGGAAAACAAACTAAAGCGCCCT
TGTGGCGCTTTAGTTT

A.1.2. RyjAΔ2 deleted 50 nt - 96 nt

ATCAACACCAACCGGAACCTCCACCACGTGCTCGAATGAGGTGTGTTGAAAGACCGGAAAAC
AAACTAAAGCGCCCTTGTGGCGCTTTAGTTT

A.1.3. RyjAΔ3 deleted 110 nt -140 nt

ATCAACACCAACCGGAACCTCCACCACGTGCTCGAATGAGGTGTGTTGACGTCGGGGGAAAC
CCTCCTGTGTACCAGCGGGATAGAGAGAAAGACAAAGACCGGAAAACA

A.1.4. RyjAS3 substituted 110 nt -140 nt

ATCAACACCAACCGGAACCTCCACCACGTGCTCGAATGAGGTGTGTTGACGTCGGGGGAAAC
CCTCCTGTGTACCAGCGGGATAGAGAGAAAGACAAAGACCGGAAAACAAAACAATACGCGGC
TTCGCCGCGTATTGTTTTGTTCATCTT

A.1.5. IsrC

>gi|49175990:2069339-2069542 Escherichia coli str. K-12 substr. MG1655 chromosome, complete genome

ACGATCAATATCTATTTTATCGATCGTTTATATCGATCGATAAGCTAATAATAACCTTTGTC
AGAACATGCACAGATACGTACAGAAAGACATTCAGGGAACAACAGAACCACAATTCAGAAAC
TCCCACAGCCGGACCTCCGGCACTGTAACCCTTTACCTGCCGGTATCCACGTTTGTGGGTAC
CGGCTTTTTTATTCACC

A.2. Sequencing results

Single pass analysis at Bangalore Genei Pvt. Ltd, India (now Merck Lifesciences, India) using M13 sequencing primers was done for sequencing the clones constructed in this study.

A.2.1. pRyjA

TAAAAGACGATCGAGCTCAGGCGCGCCTTATTAAGCTGAGATCCTGAAAGTCGCTATAGCTG
GAAGATGAACAAACTCAAGCGCCACAAAGAGAGCGCTTTAGTTTGTTTTCCGGTCTTTGTCT
TTCTCTCTATCCCGCCTGGTACACAGGAGGGTTTCCCCCGACGTCAACACACCTCATTCGAG
CACGTGGTGGAGGTTCCGGTTGGTGTTGATATGTCTCCTCAGCGTTTAAACCCTGCAGGAAG
CTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCAC

ACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTC
ACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCA
TTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCT
CGCTCACTGACTCCCTGCGCTCGGTCGTTCGGCTGCG

A.2.2. pAntiIsrC

GGCGTATACGACCACTATAGGGCGAATACATTGCTCCTTCAGAATTGGGCGGGGCTCGTACT
CAGTGGATGGAATCCGTGCAATAGCTCAATAATAGAATAAAACGATCAATATCTATTATATC
GATCGTTTATATAAGATCGATAAGCTAATAATAACCTTTGTCAGTAACATGCACAGATACGT
ACAGAAAGACATTCAGGGAACAACAGAACCACAATTCAGAAACTCCCACAGCCGGACCCCCG
GCACTGTAACCCTTTACCTGCCGGTATCCACGTTTGTGGGTACCGGCTTTTTTATTCACCCT
CAATCTAAGGAAAAGCTGATGAAACGACATCTGAAGCTTATCGATACCGTCGACCTCGAGGG
GGGGCGCCGCACCCAGCTTTTGTTCCCTTTAGTGAGGGTTAATTGCGCGCTTGGCGTAATCA
TGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGC
CGGGAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGT
TGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGC
CAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTC
GCTGCGCTCGGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGG
TTATCCACAGAATCAGGGATAACGCAGGAAAAACATGTGAGCAAAGCCAGCAAAGCCAGGAA
CCGTAAAAGCCCGTTGCTGGCTTTTTTCATAGCTTCCCCCCCTGAAGAGCATCCAAAATTGA
CG

A.2.3. pRyjAΔ2

CGTAGGCGGGACGACGACTCAGGGCGCTTTAGTTTGTTTTCCGGTCTTTCAACACACCTCAT
TCGAGCACGTGGTGGAGGTTCCGGTTGGCGAAACTAAAGCGCCACAAGGGCGCTTTAGTTTG
TTTTCCGGTCTTTCAACACACCTCATTCGAGCACGTGGTGGAGGTTCCGGTTGGTGTTGATT
GCACTGCAGGAAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCG
CTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATG
AGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGT
CGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGC
TCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATC
AGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACA
TGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAGGCCGCGTTGCTGGCGTTTTTCC
ATAGGCTCCGCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAAAGTGCGAAACCCG
ACAGACTATAAGATACCAGGCGTTTCCCCTGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACC
CTGCCGCTACCGGATACCTGTCCGCCTTCTCCTTCGGGAACCTTG

A.2.4. pRyjAΔ3

CGATAAAAAAGACAACGACTCGTGTTTTCCGGTCTTTGTCTTTCTCTCTATCCCGCTGGTAC
ACAGGAGGGTTTCCCCCGACGTCAACACACCTCATTCGAGCACGTGGTGGAGGTTCCGGTTG
GTGTTGATTGCACCGTGTTTTCCGGTCTTTGTCTTTCTCTCTATCCCGCTGGTACACAGGAG
GGTTTCCCCCGACGTCAACACACCTCATTCGAGCACGTGGTGGAGGTTCCGGTTGGTGTTGA
TTGCACTGCAGGAAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATC
CGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAA
TGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCT
GTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGC
GCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTA
TCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAAAC

ATGTGAGCAAAGGCCAGCAAAGCCAGGAACCGTAAAAGGCCGCGTTGCTGGCGTTTTCATAG
CTCGCCCCCTGACGAGCATCACAAAATCGACGCTCAGCGAGGTGGCGAACCCGAAGGATATA
AGATACAGCGTTCCCTGAAGCTCCTCGTGCGCTCTCTGTTC

A.2.5. pRyjAS3

TGNNCCATCTTTCTGGTTTGCTCTTTTATTCAACACCAACCGGGACCGCCCCAGGTGCTCGA
NTGAGGTGTGGTGACGTCGGGGGGAACCCTCCTGGGGCCAGGGGGATAGAGAGAAAGACAAA
GACCGGAAAACAAAACAATACGCGGCTTCGCCGCGTATTGTTTTGTTCATCTTCGATCTCGA
ATTCACTGGGCGTCGTTTTACAACGTCGGGACTGGGAAAACCCTGGCGTTACCCGGCTTAAT
CGCCTTGCAGCACATCCCCCTTTCGCCGGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCG
CCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGCGCCTGATGCGGTATTTTCTCCTTA
CGCATCTGTGCGGTATTTCACACCGCATATGGTGCACTCTCAGTACAATCTGCTCTGATGCC
GCATAGTTAAGCCAGCCCCGACACCCGCCAACACCCGCTGACGCGCCCTGACGGGCTTGTCT
GCTCCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTCAGAGGT
TTTCACCGTCATCACCGAAACGCGCGAGACGAAAGGGCCTCGTGATACGCCTATTTTTATAG
GTTAATGTCATGATAATAATGGTTTCTTAGACGTCAGGTGGCACTTTTCGGGGAAATGTGCG
CGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAAT
AACCCTGATAAATGCTTCAATAATCTGCGGTAAAGCTCATCAGCGTGGTCGTGAAGCGATTT
CACAGATGTCTGCCTGTTCATCCGCGTCCAGCTCGTTGAGTTTCTCCAGAAGCGTTAATGTC
TGGCTTCTGATAAGCGGGCCATGTTAAGGGCCGGTTTTTTCCTGTTGGTCACTGATGCCTCC
GTGTAAGGGGGATTTCTGTTCATGGGGGTAATGATACCGATGAAACGAGAGAGGATGCTCAC
GATACGGG

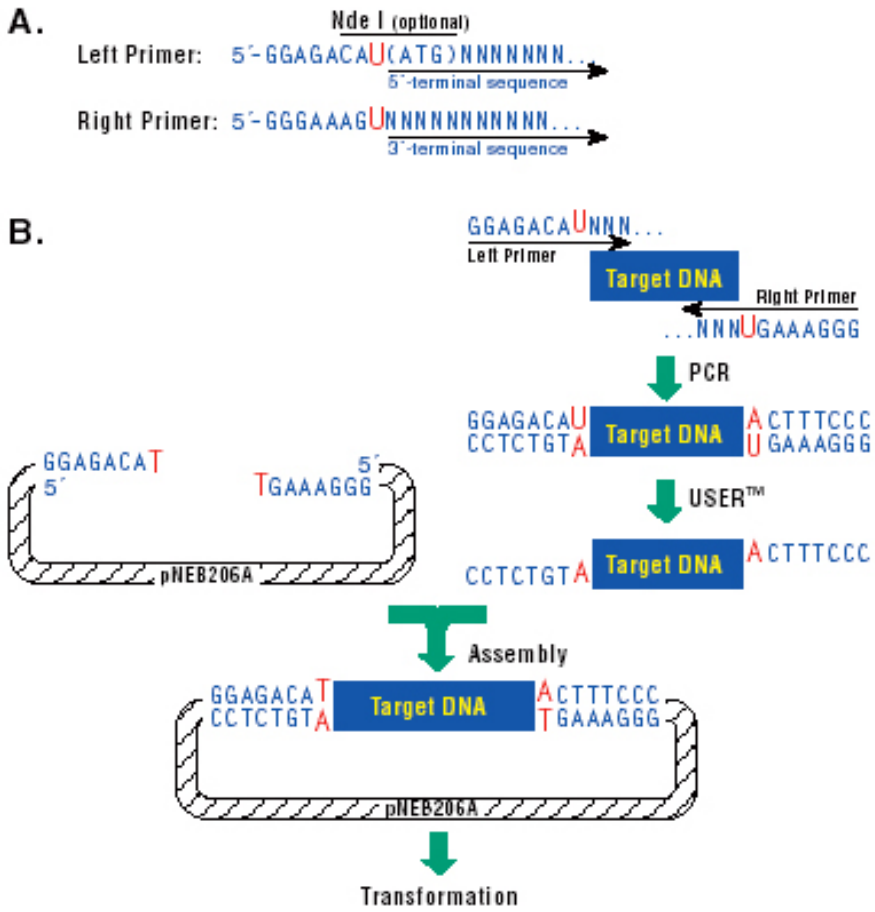
A.3. Sequence and restriction maps of plasmids

A.3.1. pNEB206A (supplied as linearized, New England Biolabs, Cat. No. E5500S)

pNEB206A is an *E. coli* cloning vector of about 2722 bp that allows blue-white selection of clones. It has a *lac* promoter, pUC origin of replication and ampicillin resistance. The eight nucleotide single-strand extensions on the linear plasmid facilitate easy cloning of genes into it.

A. Design strategy of pNEB206A vector compatible primers

B. PCR product cloning method using the USER friendly cloning kit.

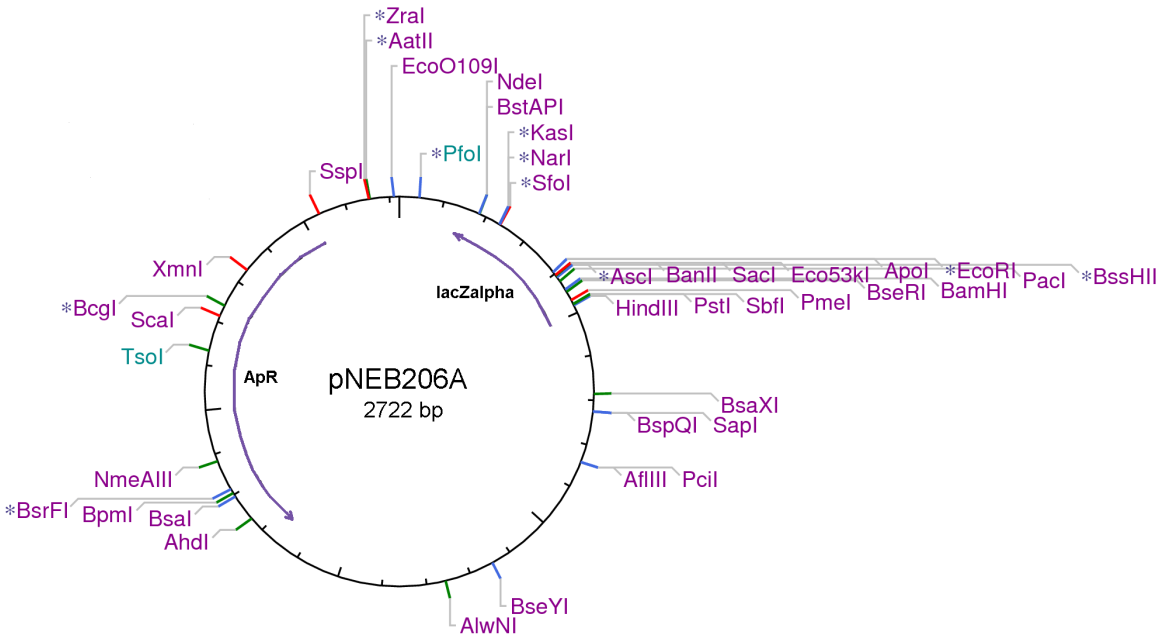


A.3.1.1. Features: 2722 bp, ampicillin resistance marker

505- 146	*lacZ* alpha coding sequence (CDS) (start 493, complementary strand)
555- 550	P*lac* promoter -10 sequence (TATGTT)
579- 574	P*lac* promoter -35 sequence (TTTACA)
611- 599	CAP protein binding site
393- 488	multiple cloning site (EcoRI-HindIII)
438- 461	top strand deleted by XbaI + N.BbvCIB digestion
430- 453	bottom strand deleted by XbaI + N.BbvCIB digestion
1491- 903	Ori (anticlockwise) (RNAII -35 to RNA/DNA switch point):
1309-1314	RNAI transcript promoter -35 sequence (TTGAAG)
1331-1336	RNAI transcript promoter -10 sequence (GCTACA)
1345-1452	RNAI transcript
1455- 903	RNAII transcript (complementary strand)
1470-1465	RNAII transcript promoter -10 sequence (CGTAAT)

1491-1486	RNAII transcript promoter -35 sequence (TTGAGA)
2522-1662	beta-lactamase (bla; amp-r) CDS (start 2522, complementary strand)
2522-2454	beta-lactamase signal peptide CDS (start 2522, complementary strand)

A.3.1.2. Restriction map of pNEB206A



A.3.2. pBluescript II phagemids

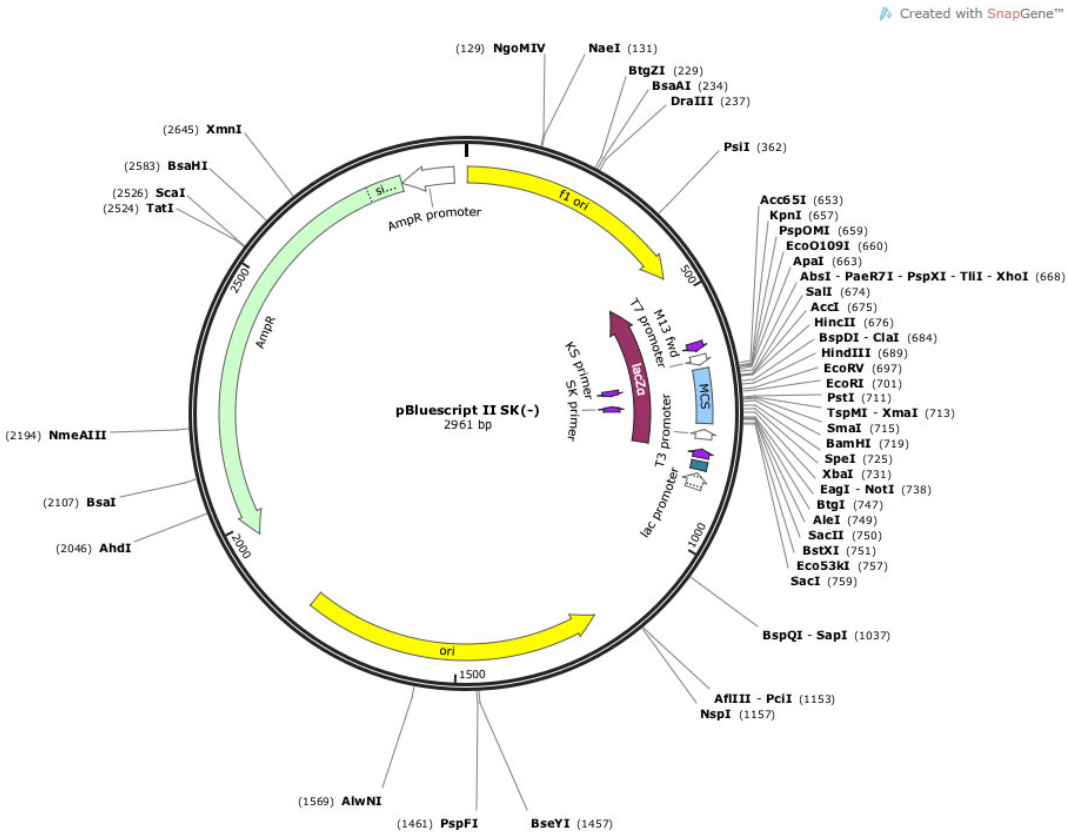
The pBluescript II phagemids (plasmids with a phage origin) are cloning vectors. pBluescript II (+) and (–) are available with two polylinker orientations designated as either KS or SK using the following convention: (1) in the KS orientation, the *Kpn* I restriction site is nearest the *lac*Z promoter and the *Sac* I restriction site is farthest from the *lac*Z promoter; and (2) in the SK orientation, the *Sac* I site is the closest restriction site to the *lac*Z promoter and the *Kpn* I site is the farthest

A.3.2.1 pBluescript II SK (+/-)

Feature Nucleotide Position

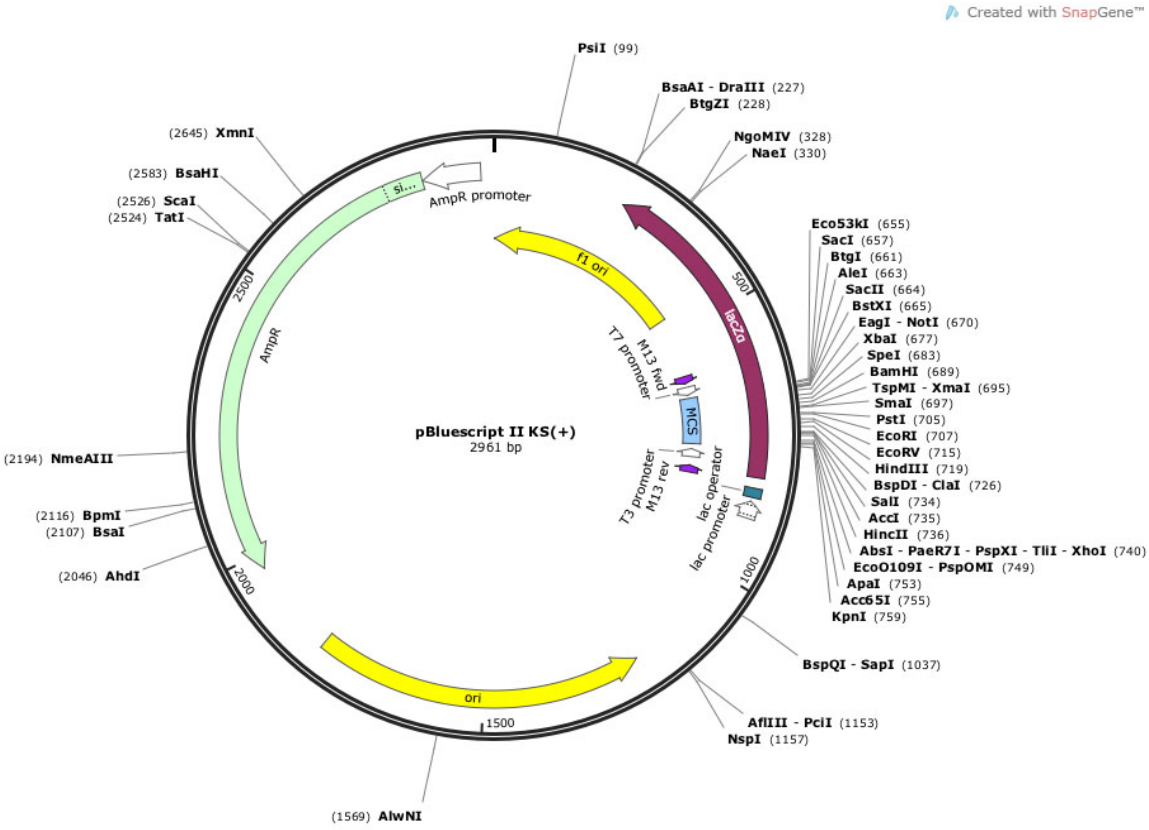
135–441	f1 (+) origin of ss-DNA replication [pBluescript SK (+) only]
21–327f1	(–) origin of ss-DNA replication [pBluescript SK (–) only]

460–816	β-galactosidase α-fragment coding sequence (lacZ')
653–760	multiple cloning site
643	T7 promoter transcription initiation site
774	T3 promoter transcription initiation site
817–938	*lac* promoter
1158–1825	pUC origin of replication
1976–2833	ampicillin resistance (*bla*) ORF



A.3.2.2. pBluescript II KS (+/-)

Feature Nucleotide Position	
135–441	f1 (+) origin of ss-DNA replication [pBluescript KS (+) only]
21–327f1	(–) origin of ss-DNA replication [pBluescript KS (–) only]
460–816	β-galactosidase α-fragment coding sequence (lacZ')
653–760	multiple cloning site
643	T7 promoter transcription initiation site
774	T3 promoter transcription initiation site
817–938	*lac* promoter
1158–1825	pUC origin of replication
1976–2833	ampicillin resistance (*bla*) ORF


Created with SnapGene™
pBluescript II KS(+)
2961 bp
PsiI (99)
BsaAI - DraIII (227)
BtgZI (228)
NgoMIV (328)
NaeI (330)
Eco53kI (655)
SacI (657)
BtgI (661)
AleI (663)
SacII (664)
BstXI (665)
EagI - NotI (670)
XbaI (677)
SpeI (683)
BamHI (689)
TspMI - XmaI (695)
SmaI (697)
PstI (705)
EcoRI (707)
EcoRV (715)
HindIII (719)
BspDI - ClaI (726)
SalI (734)
AccI (735)
HincII (736)
AbsI - PaeR7I - PspXI - TliI - XhoI (740)
EcoO109I - PspOMI (749)
ApaI (753)
Acc65I (755)
KpnI (759)
BspQI - SapI (1037)
AflIII - PciI (1153)
NspI (1157)
(1569) AlwNI
(2194) NmeAIII
(2116) BpmI
(2107) BsaI
(2046) AhdI
(2645) XmnI
(2583) BsaHI
(2526) ScaI
(2524) TatI
AmpR promoter
AmpR
f1 ori
lacZα
T7 promoter
M13 fwd
MCS
T3 promoter
M13 rev
lac operator
lac promoter
ori
500
1000
1500
2000
2500

References

Aarons, S., Abbas, A., Adams, C., Fenton, A. & O'Gara, F. (2000). A regulatory RNA (PrrB RNA) modulates expression of secondary metabolite genes in Pseudomonas fluorescens F113. *J Bacteriol* **182**, 3913-3919.

Aiba, H., Matsuyama, S., Mizuno, T. & Mizushima, S. (1987). Function of micF as an antisense RNA in osmoregulatory expression of the ompF gene in Escherichia coli. *J Bacteriol* **169**, 3007-3012.

Aiba, H. (2007). Mechanism of RNA silencing by Hfq-binding small RNAs. *Curr Opin Microbiol* **10**, 134-139.

Alberts, B. (2008). Molecular Biology of the Cell. *New York: Garland Science* **Fifth Edition**.

Altuvia, S., Weinstein-Fischer, D., Zhang, A., Postow, L. & Storz, G. (1997). A small, stable RNA induced by oxidative stress: role as a pleiotropic regulator and antimutator. *Cell* **90**, 43-53.

Altuvia, S., Zhang, A., Argaman, L., Tiwari, A. & Storz, G. (1998). The Escherichia coli OxyS regulatory RNA represses fhlA translation by blocking ribosome binding. *EMBO J* **17**, 6069-6075.

Amabile-Cuevas, C. F. & Demple, B. (1991). Molecular characterization of the soxRS genes of Escherichia coli: two genes control a superoxide stress regulon. *Nucleic Acids Res* **19**, 4479-4484.

Andersen, J., Forst, S. A., Zhao, K., Inouye, M. & Delihas, N. (1989). The function of micF RNA. micF RNA is a major factor in the thermal regulation of OmpF protein in Escherichia coli. *J Biol Chem* **264**, 17961-17970.

Andersen, J. & Delihas, N. (1990). micF RNA binds to the 5' end of ompF mRNA and to a protein from Escherichia coli. *Biochemistry* **29**, 9249-9256.

Ando, Y., Asari, S., Suzuma, S., Yamane, K. & Nakamura, K. (2002). Expression of a small RNA, BS203 RNA, from the yocI-yocJ intergenic region of Bacillus subtilis genome. *FEMS Microbiol Lett* **207**, 29-33.

Antal, M., Bordeau, V., Douchin, V. & Felden, B. (2005). A small bacterial RNA regulates a putative ABC transporter. *J Biol Chem* **280**, 7901-7908.

Aravin, A. A., Lagos-Quintana, M., Yalcin, A., Zavolan, M., Marks, D., Snyder, B., Gaasterland, T., Meyer, J. & Tuschl, T. (2003). The small RNA profile during Drosophila melanogaster development. *Dev Cell* **5**, 337-350.

Aravin, A. A., Sachidanandam, R., Bourc'his, D., Schaefer, C., Pezic, D., Toth, K. F., Bestor, T. & Hannon, G. J. (2008). A piRNA pathway primed by individual transposons is linked to de novo DNA methylation in mice. *Mol Cell* **31**, 785-799.

Argaman, L. & Altuvia, S. (2000). fhlA repression by OxyS RNA: kissing complex formation at two sites results in a stable antisense-target RNA complex. *J Mol Biol* **300**, 1101-1112.

Argaman, L., Hershberg, R., Vogel, J., Bejerano, G., Wagner, E. G., Margalit, H. & Altuvia, S. (2001). Novel small RNA-encoding genes in the intergenic regions of Escherichia coli. *Curr Biol* **11**, 941-950.

Arnvig, K. B. & Young, D. B. (2009). Identification of small RNAs in Mycobacterium tuberculosis. *Mol Microbiol* **73**, 397-408.

Artsimovitch, I. & Landick, R. (2002). The transcriptional regulator RfaH stimulates RNA chain synthesis after recruitment to elongation complexes by the exposed nontemplate DNA strand. *Cell* **109**, 193-203.

Babitzke, P. & Romeo, T. (2007). CsrB sRNA family: sequestration of RNA-binding regulatory proteins. *Curr Opin Microbiol* **10**, 156-163.

Bartolomei, M. S., Zemel, S. & Tilghman, S. M. (1991). Parental imprinting of the mouse H19 gene. *Nature* **351**, 153-155.

Bastet, L., Dube, A., Masse, E. & Lafontaine, D. A. (2011). New insights into riboswitch regulation mechanisms. *Mol Microbiol* **80**, 1148-1154.

Beloin, C., Michaelis, K., Lindner, K., Landini, P., Hacker, J., Ghigo, J. M. & Dobrindt, U. (2006). The transcriptional antiterminator RfaH represses biofilm formation in Escherichia coli. *J Bacteriol* **188**, 1316-1331.

Benne, R., Van den Burg, J., Brakenhoff, J. P., Sloof, P., Van Boom, J. H. & Tromp, M. C. (1986). Major transcript of the frameshifted coxII gene from trypanosome mitochondria contains four nucleotides that are not encoded in the DNA. *Cell* **46**, 819-826.

Bintu, L., Buchler, N. E., Garcia, H. G., Gerland, U., Hwa, T., Kondev, J., Kuhlman, T. & Phillips, R. (2005a). Transcriptional regulation by the numbers: applications. *Curr Opin Genet Dev* **15**, 125-135.

Bintu, L., Buchler, N. E., Garcia, H. G., Gerland, U., Hwa, T., Kondev, J. & Phillips, R. (2005b). Transcriptional regulation by the numbers: models. *Curr Opin Genet Dev* **15**, 116-124.

Blanchard, J. L., Wholey, W. Y., Conlon, E. M. & Pomposiello, P. J. (2007). Rapid changes in gene expression dynamics in response to superoxide reveal SoxRS-dependent and independent transcriptional networks. *PLoS One* **2**, e1186.

Blattner, F. R., Plunkett, G., 3rd, Bloch, C. A. & other authors (1997). The complete genome sequence of Escherichia coli K-12. *Science* **277**, 1453-1462.

Blumenthal, T. & Carmichael, G. G. (1979). RNA replication: function and structure of Qbeta-replicase. *Annu Rev Biochem* **48**, 525-548.

Bohn, C., Rigoulay, C., Chabelskaya, S. & other authors (2010). Experimental discovery of small RNAs in Staphylococcus aureus reveals a riboregulator of central metabolism. *Nucleic Acids Res* **38**, 6620-6636.

Boisset, S., Geissmann, T., Huntzinger, E. & other authors (2007). Staphylococcus aureus RNAIII coordinately represses the synthesis of virulence factors and the transcription regulator Rot by an antisense mechanism. *Genes Dev* **21**, 1353-1366.

Bouche, F. & Bouche, J. P. (1989). Genetic evidence that DicF, a second division inhibitor encoded by the Escherichia coli dicB operon, is probably RNA. *Mol Microbiol* **3**, 991-994.

Brannan, C. I., Dees, E. C., Ingram, R. S. & Tilghman, S. M. (1990). The product of the H19 gene may function as an RNA. *Mol Cell Biol* **10**, 28-36.

Brantl, S. (2002). Antisense RNAs in plasmids: control of replication and maintenance. *Plasmid* **48**, 165-173.

Brantl, S. (2007). Regulatory mechanisms employed by cis-encoded antisense RNAs. *Curr Opin Microbiol* **10**, 102-109.

Breaker, R. R. (2011). Prospects for riboswitch discovery and analysis. *Mol Cell* **43**, 867-879.

Breaker, R. R. (2012). Riboswitches and the RNA world. *Cold Spring Harb Perspect Biol* **4**.

Brennan, R. G. & Link, T. M. (2007). Hfq structure, function and ligand binding. *Curr Opin Microbiol* **10**, 125-133.

Britten, R. J. & Davidson, E. H. (1969). Gene regulation for higher cells: a theory. *Science* **165**, 349-357.

Brown, L. & Elliott, T. (1997). Mutations that increase expression of the rpoS gene and decrease its dependence on hfq function in Salmonella typhimurium. *J Bacteriol* **179**, 656-662.

Bushati, N. & Cohen, S. M. (2007). microRNA functions. *Annu Rev Cell Dev Biol* **23**, 175-205.

Cai, X., Hagedorn, C. H. & Cullen, B. R. (2004). Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. *RNA* **10**, 1957-1966.

Carthew, R. W. & Sontheimer, E. J. (2009). Origins and Mechanisms of miRNAs and siRNAs. *Cell* **136**, 642-655.

Chabelskaya, S., Gaillot, O. & Felden, B. (2010). A Staphylococcus aureus small RNA is required for bacterial virulence and regulates the expression of an immune-evasion molecule. *PLoS Pathog* **6**, e1000927.

Chen, S., Lesnik, E. A., Hall, T. A., Sampath, R., Griffey, R. H., Ecker, D. J. & Blyn, L. B. (2002). A bioinformatics based approach to discover small RNA genes in the Escherichia coli genome. *Biosystems* **65**, 157-177.

Chou, J. H., Greenberg, J. T. & Demple, B. (1993). Posttranscriptional repression of Escherichia coli OmpF protein in response to redox stress: positive control of the micF antisense RNA by the soxRS locus. *J Bacteriol* **175**, 1026-1031.

Christiansen, J. K., Nielsen, J. S., Ebersbach, T., Valentin-Hansen, P., Sogaard-Andersen, L. & Kallipolitis, B. H. (2006). Identification of small Hfq-binding RNAs in Listeria monocytogenes. *RNA* **12**, 1383-1396.

Coornaert, A., Lu, A., Mandin, P., Springer, M., Gottesman, S. & Guillier, M. (2010). MicA sRNA links the PhoP regulon to cell envelope stress. *Mol Microbiol* **76**, 467-479.

Coppins, R. L., Hall, K. B. & Groisman, E. A. (2007). The intricate world of riboswitches. *Curr Opin Microbiol* **10**, 176-181.

Cromie, M. J., Shi, Y., Latifi, T. & Groisman, E. A. (2006). An RNA sensor for intracellular Mg(2+). *Cell* **125**, 71-84.

Diderichsen, B. (1980). flu, a metastable gene controlling surface properties of Escherichia coli. *J Bacteriol* **141**, 858-867.

Ding, H., Hidalgo, E. & Demple, B. (1996). The redox state of the [2Fe-2S] clusters in SoxR protein regulates its activity as a transcription factor. *J Biol Chem* **271**, 33173-33175.

Ding, Y., Chan, C. Y. & Lawrence, C. E. (2004). Sfold web server for statistical folding and rational design of nucleic acids. *Nucleic Acids Res* **32**, W135-141.

Djordjevic, M. & Severinov, K. (2012). CRISPR transcript processing: a mechanism for generating a large number of small interfering RNAs. *Biol Direct* **7**, 24.

Dornenburg, J. E., Devita, A. M., Palumbo, M. J. & Wade, J. T. (2010). Widespread antisense transcription in Escherichia coli. *MBio* **1**.

Duhring, U., Axmann, I. M., Hess, W. R. & Wilde, A. (2006). An internal antisense RNA regulates expression of the photosynthesis gene isiA. *Proc Natl Acad Sci U S A* **103**, 7054-7058.

Eddy, S. R. (2001). Non-coding RNA genes and the modern RNA world. *Nat Rev Genet* **2**, 919-929.

Eliceiri, G. L. (2006). The vertebrate E1/U17 small nucleolar ribonucleoprotein particle. *J Cell Biochem* **98**, 486-495.

Engels, J. W. (2012). Gene silencing by chemically modified siRNAs. *N Biotechnol*.

Fender, A., Elf, J., Hampel, K., Zimmermann, B. & Wagner, E. G. (2010). RNAs actively cycle on the Sm-like protein Hfq. *Genes Dev* **24**, 2621-2626.

Ferre-D'Amare, A. R. & Scott, W. G. (2010). Small self-cleaving ribozymes. *Cold Spring Harb Perspect Biol* **2**, a003574.

Fexby, S., Bjarnsholt, T., Jensen, P. O., Roos, V., Hoiby, N., Givskov, M. & Klemm, P. (2007). Biological Trojan horse: Antigen 43 provides specific bacterial uptake and survival in human neutrophils. *Infect Immun* **75**, 30-34.

Filiatrault, M. J., Stodghill, P. V., Bronstein, P. A. & other authors (2010). Transcriptome analysis of Pseudomonas syringae identifies new genes, noncoding RNAs, and antisense activity. *J Bacteriol* **192**, 2359-2372.

Folichon, M., Arluison, V., Pellegrini, O., Huntzinger, E., Regnier, P. & Hajnsdorf, E. (2003). The poly(A) binding protein Hfq protects RNA from RNase E and exoribonucleolytic degradation. *Nucleic Acids Res* **31**, 7302-7310.

Fozo, E. M., Kawano, M., Fontaine, F., Kaya, Y., Mendieta, K. S., Jones, K. L., Ocampo, A., Rudd, K. E. & Storz, G. (2008). Repression of small toxic protein synthesis by the Sib and OhsC small RNAs. *Mol Microbiol* **70**, 1076-1093.

Frohlich, K. S. & Vogel, J. (2009). Activation of gene expression by small RNA. *Curr Opin Microbiol* **12**, 674-682.

Gabory, A., Ripoche, M. A., Le Digarcher, A. & other authors (2009). H19 acts as a trans regulator of the imprinted gene network controlling growth in mice. *Development* **136**, 3413-3421.

Garst, A. D. & Batey, R. T. (2009). A switch in time: detailing the life of a riboswitch. *Biochim Biophys Acta* **1789**, 584-591.

Gaudu, P. & Weiss, B. (1996). SoxR, a [2Fe-2S] transcription factor, is active only in its oxidized form. *Proc Natl Acad Sci U S A* **93**, 10094-10098.

Gaudu, P. & Weiss, B. (2000). Flavodoxin mutants of Escherichia coli K-12. *J Bacteriol* **182**, 1788-1793.

Geissmann, T., Chevalier, C., Cros, M. J. & other authors (2009). A search for small noncoding RNAs in Staphylococcus aureus reveals a conserved sequence motif for regulation. *Nucleic Acids Res* **37**, 7239-7257.

Georg, J. & Hess, W. R. (2011). cis-antisense RNA, another level of gene regulation in bacteria. *Microbiol Mol Biol Rev* **75**, 286-300.

Gilbert, W. (1986). Origin of life: The RNA world. *Nature* **319**, 618–618.

Gomez-Lozano, M., Marvig, R. L., Molin, S. & Long, K. S. (2012). Genome-wide identification of novel small RNAs in Pseudomonas aeruginosa. *Environ Microbiol* **14**, 2006-2016.

Gonzalez, N., Heeb, S., Valverde, C., Kay, E., Reimmann, C., Junier, T. & Haas, D. (2008). Genome-wide search reveals a novel GacA-regulated small RNA in Pseudomonas species. *BMC Genomics* **9**, 167.

Gorke, B. & Vogel, J. (2008). Noncoding RNA control of the making and breaking of sugars. *Genes Dev* **22**, 2914-2925.

Gott, J. M. & Emeson, R. B. (2000). Functions and mechanisms of RNA editing. *Annu Rev Genet* **34**, 499-531.

Gottesman, S. (2005). Micros for microbes: non-coding regulatory RNAs in bacteria. *Trends Genet* **21**, 399-404.

Greenberg, J. T., Monach, P., Chou, J. H., Josephy, P. D. & Demple, B. (1990). Positive control of a global antioxidant defense regulon activated by superoxide-generating agents in Escherichia coli. *Proc Natl Acad Sci U S A* **87**, 6181-6185.

Griffin, B. E. (1971). Separation of 32P-labelled ribonucleic acid components. The use of polyethylenimine-cellulose (TLC) as a second dimension in separating oligoribonucleotides of '4.5 S' and 5 S from E. coli. *FEBS Lett* **15**, 165-168.

Gu, M. & Imlay, J. A. (2011). The SoxRS response of Escherichia coli is directly activated by redox-cycling drugs rather than by superoxide. *Mol Microbiol* **79**, 1136-1150.

Guigueno, A., Dassa, J., Belin, P. & Boquet, P. L. (2001). Oversynthesis of a new Escherichia coli small RNA suppresses export toxicity of DsbA'-PhoA unfoldable periplasmic proteins. *J Bacteriol* **183**, 1147-1158.

Guillier, M. & Gottesman, S. (2006). Remodelling of the Escherichia coli outer membrane by two small regulatory RNAs. *Mol Microbiol* **59**, 231-247.

Hagan, E. C. & Mobley, H. L. (2007). Uropathogenic Escherichia coli outer membrane antigens expressed during urinary tract infection. *Infect Immun* **75**, 3941-3949.

Hasman, H., Chakraborty, T. & Klemm, P. (1999). Antigen-43-mediated autoaggregation of Escherichia coli is blocked by fimbriation. *J Bacteriol* **181**, 4834-4841.

Hebrard, M., Kroger, C., Srikumar, S., Colgan, A., Handler, K. & Hinton, J. (2012). sRNAs and the virulence of Salmonella enterica serovar Typhimurium. *RNA Biol* **9**.

Heeb, S., Blumer, C. & Haas, D. (2002). Regulatory RNA as mediator in GacA/RsmA-dependent global control of exoproduct formation in Pseudomonas fluorescens CHA0. *J Bacteriol* **184**, 1046-1056.

Heidrich, N., Moll, I. & Brantl, S. (2007). In vitro analysis of the interaction between the small RNA SR1 and its primary target ahrC mRNA. *Nucleic Acids Res* **35**, 4331-4346.

Henderson, I. R., Meehan, M. & Owen, P. (1997a). A novel regulatory mechanism for a novel phase-variable outer membrane protein of Escherichia coli. *Adv Exp Med Biol* **412**, 349-355.

Henderson, I. R., Meehan, M. & Owen, P. (1997b). Antigen 43, a phase-variable bipartite outer membrane protein, determines colony morphology and autoaggregation in Escherichia coli K-12. *FEMS Microbiol Lett* **149**, 115-120.

Henderson, I. R. & Owen, P. (1999). The major phase-variable outer membrane protein of Escherichia coli structurally resembles the immunoglobulin A1 protease class of exported protein and is regulated by a novel mechanism involving Dam and oxyR. *J Bacteriol* **181**, 2132-2141.

Hermsen, R., Ursem, B. & ten Wolde, P. R. (2010). Combinatorial gene regulation using auto-regulation. *PLoS Comput Biol* **6**, e1000813.

Hershberg, R., Altuvia, S. & Margalit, H. (2003). A survey of small RNA-encoding genes in Escherichia coli. *Nucleic Acids Res* **31**, 1813-1820.

Heurlier, K., Williams, F., Heeb, S., Dormond, C., Pessi, G., Singer, D., Camara, M., Williams, P. & Haas, D. (2004). Positive control of swarming, rhamnolipid synthesis, and lipase production by the posttranscriptional RsmA/RsmZ system in Pseudomonas aeruginosa PAO1. *J Bacteriol* **186**, 2936-2945.

Hidalgo, E. & Demple, B. (1994). An iron-sulfur center essential for transcriptional activation by the redox-sensing SoxR protein. *EMBO J* **13**, 138-146.

Hidalgo, E., Leautaud, V. & Demple, B. (1998). The redox-regulated SoxR protein acts from a single DNA site as a repressor and an allosteric activator. *EMBO J* **17**, 2629-2636.

Hindley, J. (1967). Fractionation of 32P-labelled ribonucleic acids on polyacrylamide gels and their characterization by fingerprinting. *J Mol Biol* **30**, 125-136.

Hollands, K., Proshkin, S., Sklyarova, S., Epshtein, V., Mironov, A., Nudler, E. & Groisman, E. A. (2012). Riboswitch control of Rho-dependent transcription termination. *Proc Natl Acad Sci U S A* **109**, 5376-5381.

Hopkins, J. F., Panja, S. & Woodson, S. A. (2011). Rapid binding and release of Hfq from ternary complexes during RNA annealing. *Nucleic Acids Res* **39**, 5193-5202.

Hopper, A. K. (2006). Cellular dynamics of small RNAs. *Crit Rev Biochem Mol Biol* **41**, 3-19.

Hu, Z., Zhang, A., Storz, G., Gottesman, S. & Leppla, S. H. (2006). An antibody-based microarray assay for small RNA detection. *Nucleic Acids Res* **34**, e52.

Huntzinger, E., Boisset, S., Saveanu, C. & other authors (2005). Staphylococcus aureus RNAIII and the endoribonuclease III coordinately regulate spa gene expression. *EMBO J* **24**, 824-835.

Hwang, W., Arluison, V. & Hohng, S. (2011). Dynamic competition of DsrA and rpoS fragments for the proximal binding site of Hfq as a means for efficient annealing. *Nucleic Acids Res* **39**, 5131-5139.

Ikemura, T. & Dahlberg, J. E. (1973a). Small ribonucleic acids of Escherichia coli. II. Noncoordinate accumulation during stringent control. *J Biol Chem* **248**, 5033-5041.

Ikemura, T. & Dahlberg, J. E. (1973b). Small ribonucleic acids of Escherichia coli. I. Characterization by polyacrylamide gel electrophoresis and fingerprint analysis. *J Biol Chem* **248**, 5024-5032.

Inbaraj, J. J. & Chignell, C. F. (2004). Cytotoxic action of juglone and plumbagin: a mechanistic study using HaCaT keratinocytes. *Chem Res Toxicol* **17**, 55-62.

Jacob, F. & Monod, J. (1961). Genetic regulatory mechanisms in the synthesis of proteins. *J Mol Biol* **3**, 318-356.

Ji, G., Beavis, R. C. & Novick, R. P. (1995). Cell density control of staphylococcal virulence mediated by an octapeptide pheromone. *Proc Natl Acad Sci U S A* **92**, 12055-12059.

Johansen, J., Rasmussen, A. A., Overgaard, M. & Valentin-Hansen, P. (2006). Conserved small non-coding RNAs that belong to the sigmaE regulon: role in down-regulation of outer membrane proteins. *J Mol Biol* **364**, 1-8.

Jorgensen, M. G., Nielsen, J. S., Boysen, A., Franch, T., Moller-Jensen, J. & Valentin-Hansen, P. (2012). Small regulatory RNAs control the multi-cellular adhesive lifestyle of Escherichia coli. *Mol Microbiol* **84**, 36-50.

Kawamoto, H., Morita, T., Shimizu, A., Inada, T. & Aiba, H. (2005). Implication of membrane localization of target mRNA in the action of a small RNA: mechanism of post-transcriptional regulation of glucose transporter in Escherichia coli. *Genes Dev* **19**, 328-338.

Kawamoto, H., Koide, Y., Morita, T. & Aiba, H. (2006). Base-pairing requirement for RNA silencing by a bacterial small RNA and acceleration of duplex formation by Hfq. *Mol Microbiol* **61**, 1013-1022.

Kawano, M., Aravind, L. & Storz, G. (2007). An antisense RNA controls synthesis of an SOS-induced toxin evolved from an antitoxin. *Mol Microbiol* **64**, 738-754.

Keseler, I. M., Collado-Vides, J., Gama-Castro, S., Ingraham, J., Paley, S., Paulsen, I. T., Peralta-Gil, M. & Karp, P. D. (2005). EcoCyc: a comprehensive database resource for Escherichia coli. *Nucleic Acids Res* **33**, D334-337.

Kimata, K., Tanaka, Y., Inada, T. & Aiba, H. (2001). Expression of the glucose transporter gene, ptsG, is regulated at the mRNA degradation step in response to glycolytic flux in Escherichia coli. *EMBO J* **20**, 3587-3595.

Kjaergaard, K., Schembri, M. A., Hasman, H. & Klemm, P. (2000). Antigen 43 from Escherichia coli induces inter- and intraspecies cell aggregation and changes in colony morphology of Pseudomonas fluorescens. *J Bacteriol* **182**, 4789-4796.

Kjaergaard, K., Hasman, H., Schembri, M. A. & Klemm, P. (2002). Antigen 43-mediated autotransporter display, a versatile bacterial cell surface presentation system. *J Bacteriol* **184**, 4197-4204.

Klattenhoff, C. & Theurkauf, W. (2008). Biogenesis and germline functions of piRNAs. *Development* **135**, 3-9.

Klemm, P., Hjerrild, L., Gjermansen, M. & Schembri, M. A. (2004). Structure-function analysis of the self-recognizing Antigen 43 autotransporter protein from Escherichia coli. *Mol Microbiol* **51**, 283-296.

Kobayashi, E., Hornicek, F. J. & Duan, Z. (2012). MicroRNA Involvement in Osteosarcoma. *Sarcoma* **2012**, 359739.

Koo, M. S., Lee, J. H., Rah, S. Y., Yeo, W. S., Lee, J. W., Lee, K. L., Koh, Y. S., Kang, S. O. & Roe, J. H. (2003). A reducing system of the superoxide sensor SoxR in Escherichia coli. *EMBO J* **22**, 2614-2622.

Kovach, M. E., Phillips, R. W., Elzer, P. H., Roop, R. M., 2nd & Peterson, K. M. (1994). pBBR1MCS: a broad-host-range cloning vector. *Biotechniques* **16**, 800-802.

Kozak, M. (2005). Regulation of translation via mRNA structure in prokaryotes and eukaryotes. *Gene* **361**, 13-37.

Kroger, C., Dillon, S. C., Cameron, A. D. & other authors (2012). The transcriptional landscape and small RNAs of Salmonella enterica serovar Typhimurium. *Proc Natl Acad Sci U S A* **109**, E1277-1286.

Lagos-Quintana, M., Rauhut, R., Lendeckel, W. & Tuschl, T. (2001). Identification of novel genes coding for small expressed RNAs. *Science* **294**, 853-858.

Lau, N. C., Lim, L. P., Weinstein, E. G. & Bartel, D. P. (2001). An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. *Science* **294**, 858-862.

Lease, R. A., Cusick, M. E. & Belfort, M. (1998). Riboregulation in Escherichia coli: DsrA RNA acts by RNA:RNA interactions at multiple loci. *Proc Natl Acad Sci U S A* **95**, 12456-12461.

Lee, R. C. & Ambros, V. (2001). An extensive class of small RNAs in Caenorhabditis elegans. *Science* **294**, 862-864.

Lee, Y., Kim, M., Han, J., Yeom, K. H., Lee, S., Baek, S. H. & Kim, V. N. (2004). MicroRNA genes are transcribed by RNA polymerase II. *EMBO J* **23**, 4051-4060.

Lenz, D. H., Mok, K. C., Lilley, B. N., Kulkarni, R. V., Wingreen, N. S. & Bassler, B. L. (2004). The small RNA chaperone Hfq and multiple small RNAs control quorum sensing in Vibrio harveyi and Vibrio cholerae. *Cell* **118**, 69-82.

Lenz, D. H., Miller, M. B., Zhu, J., Kulkarni, R. V. & Bassler, B. L. (2005). CsrA and three redundant small RNAs regulate quorum sensing in Vibrio cholerae. *Mol Microbiol* **58**, 1186-1202.

Lippa, A. M. & Goulian, M. (2009). Feedback inhibition in the PhoQ/PhoP signaling system by a membrane peptide. *PLoS Genet* **5**, e1000788.

Liu, J. M., Livny, J., Lawrence, M. S., Kimball, M. D., Waldor, M. K. & Camilli, A. (2009). Experimental discovery of sRNAs in Vibrio cholerae by direct cloning, 5S/tRNA depletion and parallel sequencing. *Nucleic Acids Res* **37**, e46.

Liu, J. M. & Camilli, A. (2010). A broadening world of bacterial small RNAs. *Curr Opin Microbiol* **13**, 18-23.

Liu, M. Y., Gui, G., Wei, B., Preston, J. F., 3rd, Oakford, L., Yuksel, U., Giedroc, D. P. & Romeo, T. (1997). The RNA molecule CsrB binds to the global regulatory protein CsrA and antagonizes its activity in Escherichia coli. *J Biol Chem* **272**, 17502-17510.

Livny, J., Fogel, M. A., Davis, B. M. & Waldor, M. K. (2005). sRNAPredict: an integrative computational approach to identify sRNAs in bacterial genomes. *Nucleic Acids Res* **33**, 4096-4105.

Livny, J., Brencic, A., Lory, S. & Waldor, M. K. (2006). Identification of 17 Pseudomonas aeruginosa sRNAs and prediction of sRNA-encoding genes in 10 diverse pathogens using the bioinformatic tool sRNAPredict2. *Nucleic Acids Res* **34**, 3484-3493.

Livny, J., Teonadi, H., Livny, M. & Waldor, M. K. (2008). High-throughput, kingdom-wide prediction and annotation of bacterial non-coding RNAs. *PLoS One* **3**, e3197.

MacRae, I. J., Zhou, K. & Doudna, J. A. (2007). Structural determinants of RNA recognition and cleavage by Dicer. *Nat Struct Mol Biol* **14**, 934-940.

MacRae, I. J., Ma, E., Zhou, M., Robinson, C. V. & Doudna, J. A. (2008). In vitro reconstitution of the human RISC-loading complex. *Proc Natl Acad Sci U S A* **105**, 512-517.

Majdalani, N., Cunning, C., Sledjeski, D., Elliott, T. & Gottesman, S. (1998). DsrA RNA regulates translation of RpoS message by an anti-antisense mechanism, independent of its action as an antisilencer of transcription. *Proc Natl Acad Sci U S A* **95**, 12462-12467.

Majdalani, N., Chen, S., Murrow, J., St John, K. & Gottesman, S. (2001). Regulation of RpoS by a novel small RNA: the characterization of RprA. *Mol Microbiol* **39**, 1382-1394.

Majdalani, N., Hernandez, D. & Gottesman, S. (2002). Regulation and mode of action of the second small RNA activator of RpoS translation, RprA. *Mol Microbiol* **46**, 813-826.

Maki, K., Morita, T., Otaka, H. & Aiba, H. (2010). A minimal base-pairing region of a bacterial small RNA SgrS required for translational repression of ptsG mRNA. *Mol Microbiol* **76**, 782-792.

Malone, C. D. & Hannon, G. J. (2009). Small RNAs as guardians of the genome. *Cell* **136**, 656-668.

Mandin, P., Repoila, F., Vergassola, M., Geissmann, T. & Cossart, P. (2007). Identification of new noncoding RNAs in Listeria monocytogenes and prediction of mRNA targets. *Nucleic Acids Res* **35**, 962-974.

Mangan, S., Itzkovitz, S., Zaslaver, A. & Alon, U. (2006). The incoherent feed-forward loop accelerates the response-time of the gal system of Escherichia coli. *J Mol Biol* **356**, 1073-1081.

Masse, E. & Gottesman, S. (2002). A small RNA regulates the expression of genes involved in iron metabolism in Escherichia coli. *Proc Natl Acad Sci U S A* **99**, 4620-4625.

Masse, E., Escorcia, F. E. & Gottesman, S. (2003a). Coupled degradation of a small regulatory RNA and its mRNA targets in Escherichia coli. *Genes Dev* **17**, 2374-2383.

Masse, E., Majdalani, N. & Gottesman, S. (2003b). Regulatory roles for small RNAs in bacteria. *Curr Opin Microbiol* **6**, 120-124.

Masse, E., Vanderpool, C. K. & Gottesman, S. (2005). Effect of RyhB small RNA on global iron use in Escherichia coli. *J Bacteriol* **187**, 6962-6971.

Matera, A. G., Terns, R. M. & Terns, M. P. (2007). Non-coding RNAs: lessons from the small nuclear and small nucleolar RNAs. *Nat Rev Mol Cell Biol* **8**, 209-220.

Mattick, J. S. & Makunin, I. V. (2006). Non-coding RNA. *Hum Mol Genet* **15 Spec No 1**, R17-29.

Mello, C. C. & Conte, D., Jr. (2004). Revealing the world of RNA interference. *Nature* **431**, 338-342.

Mika, F., Busse, S., Possling, A., Berkholz, J., Tschowri, N., Sommerfeldt, N., Pruteanu, M. & Hengge, R. (2012). Targeting of csgD by the small regulatory RNA RprA links stationary phase, biofilm formation and cell envelope stress in Escherichia coli. *Mol Microbiol* **84**, 51-65.

Mizuno, T., Chou, M. Y. & Inouye, M. (1984). A unique mechanism regulating gene expression: translational inhibition by a complementary RNA transcript (micRNA). *Proc Natl Acad Sci U S A* **81**, 1966-1970.

Moller, T., Franch, T., Udesen, C., Gerdes, K. & Valentin-Hansen, P. (2002). Spot 42 RNA mediates discoordinate expression of the E. coli galactose operon. *Genes Dev* **16**, 1696-1706.

Moon, K. & Gottesman, S. (2009). A PhoQ/P-regulated small RNA regulates sensitivity of Escherichia coli to antimicrobial peptides. *Mol Microbiol* **74**, 1314-1330.

Morfeldt, E., Taylor, D., von Gabain, A. & Arvidson, S. (1995). Activation of alpha-toxin translation in Staphylococcus aureus by the trans-encoded antisense RNA, RNAIII. *EMBO J* **14**, 4569-4577.

Morita, T., Maki, K. & Aiba, H. (2005). RNase E-based ribonucleoprotein complexes: mechanical basis of mRNA destabilization mediated by bacterial noncoding RNAs. *Genes Dev* **19**, 2176-2186.

Morita, T., Mochizuki, Y. & Aiba, H. (2006). Translational repression is sufficient for gene silencing by bacterial small noncoding RNAs in the absence of mRNA destruction. *Proc Natl Acad Sci U S A* **103**, 4858-4863.

Morris, K. V. (2005). siRNA-mediated transcriptional gene silencing: the potential mechanism and a possible role in the histone code. *Cell Mol Life Sci* **62**, 3057-3066.

Mraheil, M. A., Billion, A., Kuenne, C. & other authors (2010). Comparative genome-wide analysis of small RNAs of major Gram-positive pathogens: from identification to application. *Microb Biotechnol* **3**, 658-676.

Mraheil, M. A., Billion, A., Mohamed, W. & other authors (2011). The intracellular sRNA transcriptome of Listeria monocytogenes during growth in macrophages. *Nucleic Acids Res* **39**, 4235-4248.

Muffler, A., Fischer, D. & Hengge-Aronis, R. (1996). The RNA-binding protein HF-I, known as a host factor for phage Qbeta RNA replication, is essential for rpoS translation in Escherichia coli. *Genes Dev* **10**, 1143-1151.

Namy, O., Rousset, J. P., Napthine, S. & Brierley, I. (2004). Reprogrammed genetic decoding in cellular gene expression. *Mol Cell* **13**, 157-168.

Nelson, P., Kiriakidou, M., Sharma, A., Maniataki, E. & Mourelatos, Z. (2003). The microRNA world: small is mighty. *Trends Biochem Sci* **28**, 534-540.

Nielsen, A. K., Thorsted, P., Thisted, T., Wagner, E. G. & Gerdes, K. (1991). The rifampicin-inducible genes srnB from F and pnd from R483 are regulated by antisense RNAs and mediate plasmid maintenance by killing of plasmid-free segregants. *Mol Microbiol* **5**, 1961-1973.

Nielsen, J. S., Larsen, M. H., Lillebaek, E. M., Bergholz, T. M., Christiansen, M. H., Boor, K. J., Wiedmann, M. & Kallipolitis, B. H. (2011). A small RNA controls expression of the chitinase ChiA in Listeria monocytogenes. *PLoS One* **6**, e19019.

Nissen, P., Hansen, J., Ban, N., Moore, P. B. & Steitz, T. A. (2000). The structural basis of ribosome activity in peptide bond synthesis. *Science* **289**, 920-930.

O'Gorman, W., Kwek, K. Y., Thomas, B. & Akoulitchev, A. (2006). Non-coding RNA in transcription initiation. *Biochem Soc Symp*, 131-140.

Opdyke, J. A., Kang, J. G. & Storz, G. (2004). GadY, a small-RNA regulator of acid response genes in Escherichia coli. *J Bacteriol* **186**, 6698-6705.

Opdyke, J. A., Fozo, E. M., Hemm, M. R. & Storz, G. (2011). RNase III participates in GadY-dependent cleavage of the gadX-gadW mRNA. *J Mol Biol* **406**, 29-43.

Padalon-Brauch, G., Hershberg, R., Elgrably-Weiss, M., Baruch, K., Rosenshine, I., Margalit, H. & Altuvia, S. (2008). Small RNAs encoded within genetic islands of Salmonella typhimurium show host-induced expression and role in virulence. *Nucleic Acids Res* **36**, 1913-1927.

Papenfort, K., Pfeiffer, V., Mika, F., Lucchini, S., Hinton, J. C. & Vogel, J. (2006). SigmaE-dependent small RNAs of Salmonella respond to membrane stress by accelerating global omp mRNA decay. *Mol Microbiol* **62**, 1674-1688.

Papenfort, K., Pfeiffer, V., Lucchini, S., Sonawane, A., Hinton, J. C. & Vogel, J. (2008). Systematic deletion of Salmonella small RNA genes identifies CyaR, a conserved CRP-dependent riboregulator of OmpX synthesis. *Mol Microbiol* **68**, 890-906.

Pellin, D., Miotto, P., Ambrosi, A., Cirillo, D. M. & Di Serio, C. (2012). A genome-wide identification analysis of small regulatory RNAs in Mycobacterium tuberculosis by RNA-Seq and conservation analysis. *PLoS One* **7**, e32723.

Pfeiffer, V., Sittka, A., Tomer, R., Tedin, K., Brinkmann, V. & Vogel, J. (2007). A small non-coding RNA of the invasion gene island (SPI-1) represses outer membrane protein synthesis from the Salmonella core genome. *Mol Microbiol* **66**, 1174-1191.

Pichon, C. & Felden, B. (2005). Small RNA genes expressed from Staphylococcus aureus genomic and pathogenicity islands with specific expression among pathogenic strains. *Proc Natl Acad Sci U S A* **102**, 14249-14254.

Pomposiello, P. J., Bennik, M. H. & Demple, B. (2001). Genome-wide transcriptional profiling of the Escherichia coli responses to superoxide stress and sodium salicylate. *J Bacteriol* **183**, 3890-3902.

Prevost, K., Salvail, H., Desnoyers, G., Jacques, J. F., Phaneuf, E. & Masse, E. (2007). The small RNA RyhB activates the translation of shiA mRNA encoding a permease of shikimate, a compound involved in siderophore synthesis. *Mol Microbiol* **64**, 1260-1273.

Prevost, K., Desnoyers, G., Jacques, J. F., Lavoie, F. & Masse, E. (2011). Small RNA-induced mRNA degradation achieved through both translation block and activated cleavage. *Genes Dev* **25**, 385-396.

Pulvermacher, S. C., Stauffer, L. T. & Stauffer, G. V. (2008). The small RNA GcvB regulates sstT mRNA expression in Escherichia coli. *J Bacteriol* **191**, 238-248.

Pulvermacher, S. C., Stauffer, L. T. & Stauffer, G. V. (2009a). Role of the sRNA GcvB in regulation of cycA in Escherichia coli. *Microbiology* **155**, 106-114.

Pulvermacher, S. C., Stauffer, L. T. & Stauffer, G. V. (2009b). Role of the Escherichia coli Hfq protein in GcvB regulation of oppA and dppA mRNAs. *Microbiology* **155**, 115-123.

Raghavan, R., Groisman, E. A. & Ochman, H. (2011). Genome-wide detection of novel regulatory RNAs in E. coli. *Genome Res* **21**, 1487-1497.

Ramani, N., Hedeshian, M. & Freundlich, M. (1994). micF antisense RNA has a major role in osmoregulation of OmpF in Escherichia coli. *J Bacteriol* **176**, 5005-5010.

Rasmussen, A. A., Eriksen, M., Gilany, K., Udesen, C., Franch, T., Petersen, C. & Valentin-Hansen, P. (2005). Regulation of ompA mRNA stability: the role of a small regulatory RNA in growth phase-dependent control. *Mol Microbiol* **58**, 1421-1429.

Reiner, R., Ben-Asouli, Y., Krilovetzky, I. & Jarrous, N. (2006). A role for the catalytic ribonucleoprotein RNase P in RNA polymerase III transcription. *Genes Dev* **20**, 1621-1635.

Reinhart, B. J., Slack, F. J., Basson, M., Pasquinelli, A. E., Bettinger, J. C., Rougvie, A. E., Horvitz, H. R. & Ruvkun, G. (2000). The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. *Nature* **403**, 901-906.

Repoila, F., Majdalani, N. & Gottesman, S. (2003). Small non-coding RNAs, co-ordinators of adaptation processes in Escherichia coli: the RpoS paradigm. *Mol Microbiol* **48**, 855-861.

Richter, A. S. & Backofen, R. (2012). Accessibility and conservation: General features of bacterial small RNA-mRNA interactions? *RNA Biol* **9**.

Rivas, E., Klein, R. J., Jones, T. A. & Eddy, S. R. (2001). Computational identification of noncoding RNAs in E. coli by comparative genomics. *Curr Biol* **11**, 1369-1373.

Roche, A., McFadden, J. & Owen, P. (2001). Antigen 43, the major phase-variable protein of the Escherichia coli outer membrane, can exist as a family of proteins encoded by multiple alleles. *Microbiology* **147**, 161-169.

Rogelj, B. (2006). Brain-specific small nucleolar RNAs. *J Mol Neurosci* **28**, 103-109.

Roth, A. & Breaker, R. R. (2009). The structural and functional diversity of metabolite-binding riboswitches. *Annu Rev Biochem* **78**, 305-334.

Rudd, K. E. (2000). EcoGene: a genome sequence database for Escherichia coli K-12. *Nucleic Acids Res* **28**, 60-64.

Saito, K., Nishida, K. M., Mori, T., Kawamura, Y., Miyoshi, K., Nagami, T., Siomi, H. & Siomi, M. C. (2006). Specific association of Piwi with rasiRNAs derived from retrotransposon and heterochromatic regions in the Drosophila genome. *Genes Dev* **20**, 2214-2222.

Sambrook, J. & Russel, D. W. (2001). Molecular cloning: A Laboratory Mannual. **Third Edition**.

Selinger, D. W., Cheung, K. J., Mei, R., Johansson, E. M., Richmond, C. S., Blattner, F. R., Lockhart, D. J. & Church, G. M. (2000). RNA expression analysis using a 30 base pair resolution Escherichia coli genome array. *Nat Biotechnol* **18**, 1262-1268.

Serganov, A. & Patel, D. J. (2007). Ribozymes, riboswitches and beyond: regulation of gene expression without proteins. *Nat Rev Genet* **8**, 776-790.

Seto, A. G., Kingston, R. E. & Lau, N. C. (2007). The coming of age for Piwi proteins. *Mol Cell* **26**, 603-609.

Sharma, C. & Heidrich, N. (2012). Small RNAs and virulence in bacterial pathogens. *RNA Biol* **9**.

Sharma, C. M., Darfeuille, F., Plantinga, T. H. & Vogel, J. (2007). A small RNA regulates multiple ABC transporter mRNAs by targeting C/A-rich elements inside and upstream of ribosome-binding sites. *Genes Dev* **21**, 2804-2817.

Sharma, C. M. & Vogel, J. (2009). Experimental approaches for the discovery and characterization of regulatory small RNA. *Curr Opin Microbiol* **12**, 536-546.

Sharma, C. M., Hoffmann, S., Darfeuille, F. & other authors (2010). The primary transcriptome of the major human pathogen Helicobacter pylori. *Nature* **464**, 250-255.

Sharma, C. M., Papenfort, K., Pernitzsch, S. R., Mollenkopf, H. J., Hinton, J. C. & Vogel, J. (2011). Pervasive post-transcriptional control of genes involved in amino acid metabolism by the Hfq-dependent GcvB small RNA. *Mol Microbiol* **81**, 1144-1165.

Sharma, C. M. & Storz, G. (2011). Interesting twists on small RNA themes in Pseudomonas aeruginosa. *Mol Microbiol* **80**, 855-859.

Sharma, C. M., Hoffmann, S., Darfeuille, F. & other authors (2012). The primary transcriptome of the major human pathogen Helicobacter pylori. *Nature* **464**, 250-255.

Shimoni, Y., Friedlander, G., Hetzroni, G., Niv, G., Altuvia, S., Biham, O. & Margalit, H. (2007). Regulation of gene expression by small non-coding RNAs: a quantitative view. *Mol Syst Biol* **3**, 138.

Shukla, G. C., Singh, J. & Barik, S. (2011). MicroRNAs: Processing, Maturation, Target Recognition and Regulatory Functions. *Mol Cell Pharmacol* **3**, 83-92.

Silvaggi, J. M., Perkins, J. B. & Losick, R. (2005). Small untranslated RNA antitoxin in Bacillus subtilis. *J Bacteriol* **187**, 6641-6650.

Simonelig, M. (2011). Developmental functions of piRNAs and transposable elements: a Drosophila point-of-view. *RNA Biol* **8**, 754-759.

Simons, R. W. & Kleckner, N. (1983). Translational control of IS10 transposition. *Cell* **34**, 683-691.

Sittka, A., Lucchini, S., Papenfort, K., Sharma, C. M., Rolle, K., Binnewies, T. T., Hinton, J. C. & Vogel, J. (2008). Deep sequencing analysis of small noncoding RNA and mRNA targets of the global post-transcriptional regulator, Hfq. *PLoS Genet* **4**, e1000163.

Slack, F. J., Basson, M., Liu, Z., Ambros, V., Horvitz, H. R. & Ruvkun, G. (2000). The lin-41 RBCC gene acts in the C. elegans heterochronic pathway between the let-7 regulatory RNA and the LIN-29 transcription factor. *Mol Cell* **5**, 659-669.

Sledjeski, D. & Gottesman, S. (1995). A small RNA acts as an antisilencer of the H-NS-silenced rcsA gene of Escherichia coli. *Proc Natl Acad Sci U S A* **92**, 2003-2007.

Sledjeski, D. D. & Gottesman, S. (1996a). Osmotic shock induction of capsule synthesis in Escherichia coli K-12. *J Bacteriol* **178**, 1204-1206.

Sledjeski, D. D., Gupta, A. & Gottesman, S. (1996b). The small RNA, DsrA, is essential for the low temperature expression of RpoS during exponential growth in Escherichia coli. *EMBO J* **15**, 3993-4000.

Song, T., Mika, F., Lindmark, B. & other authors (2008). A new Vibrio cholerae sRNA modulates colonization and affects release of outer membrane vesicles. *Mol Microbiol* **70**, 100-111.

Sonnleitner, E., Schuster, M., Sorger-Domenigg, T., Greenberg, E. P. & Blasi, U. (2006). Hfq-dependent alterations of the transcriptome profile and effects on quorum sensing in Pseudomonas aeruginosa. *Mol Microbiol* **59**, 1542-1558.

Sonnleitner, E., Sorger-Domenigg, T., Madej, M. J., Findeiss, S., Hackermuller, J., Huttenhofer, A., Stadler, P. F., Blasi, U. & Moll, I. (2008). Detection of small RNAs in

Pseudomonas aeruginosa by RNomics and structure-based bioinformatic tools. *Microbiology* **154**, 3175-3187.

Sonnleitner, E., Gonzalez, N., Sorger-Domenigg, T. & other authors (2011). The small RNA PhrS stimulates synthesis of the Pseudomonas aeruginosa quinolone signal. *Mol Microbiol* **80**, 868-885.

Soper, T., Mandin, P., Majdalani, N., Gottesman, S. & Woodson, S. A. (2010). Positive regulation by small RNAs and the role of Hfq. *Proc Natl Acad Sci U S A* **107**, 9602-9607.

Spiegelman, W. G., Reichardt, L. F., Yaniv, M., Heinemann, S. F., Kaiser, A. D. & Eisen, H. (1972). Bidirectional transcription and the regulation of Phage lambda repressor synthesis. *Proc Natl Acad Sci U S A* **69**, 3156-3160.

Storz, G., Opdyke, J. A. & Zhang, A. (2004). Controlling mRNA stability and translation with small, noncoding RNAs. *Curr Opin Microbiol* **7**, 140-144.

Stougaard, P., Molin, S. & Nordstrom, K. (1981). RNAs involved in copy-number control and incompatibility of plasmid R1. *Proc Natl Acad Sci U S A* **78**, 6008-6012.

Sutcliffe, J. G. (1979). Complete nucleotide sequence of the Escherichia coli plasmid pBR322. *Cold Spring Harb Symp Quant Biol* **43 Pt 1**, 77-90.

Talini, G., Gallori, E. & Maurel, M. C. (2009). Natural and unnatural ribozymes: back to the primordial RNA world. *Res Microbiol* **160**, 457-465.

Tetart, F., Albigot, R., Conter, A., Mulder, E. & Bouche, J. P. (1992). Involvement of FtsZ in coupling of nucleoid separation with septation. *Mol Microbiol* **6**, 621-627.

Tetart, F. & Bouche, J. P. (1992). Regulation of the expression of the cell-cycle gene ftsZ by DicF antisense RNA. Division does not require a fixed number of FtsZ molecules. *Mol Microbiol* **6**, 615-620.

Thomason, M. K., Fontaine, F., De Lay, N. & Storz, G. (2012). A small RNA that regulates motility and biofilm formation in response to changes in nutrient availability in Escherichia coli. *Mol Microbiol* **84**, 17-35.

Thompson, K. M., Rhodius, V. A. & Gottesman, S. (2007). SigmaE regulates and is regulated by a small RNA in Escherichia coli. *J Bacteriol* **189**, 4243-4256.

Tjaden, B., Saxena, R. M., Stolyar, S., Haynor, D. R., Kolker, E. & Rosenow, C. (2002). Transcriptome analysis of Escherichia coli using high-density oligonucleotide probe arrays. *Nucleic Acids Res* **30**, 3732-3738.

Tjaden, B., Goodwin, S. S., Opdyke, J. A., Guillier, M., Fu, D. X., Gottesman, S. & Storz, G. (2006). Target prediction for small, noncoding RNAs in bacteria. *Nucleic Acids Res* **34**, 2791-2802.

Toledo-Arana, A., Dussurget, O., Nikitas, G. & other authors (2009). The Listeria transcriptional landscape from saprophytism to virulence. *Nature* **459**, 950-956.

Tomizawa, J., Itoh, T., Selzer, G. & Som, T. (1981). Inhibition of ColE1 RNA primer formation by a plasmid-specified small RNA. *Proc Natl Acad Sci U S A* **78**, 1421-1425.

Trotochaud, A. E. & Wassarman, K. M. (2005). A highly conserved 6S RNA structure is required for regulation of transcription. *Nat Struct Mol Biol* **12**, 313-319.

Tsaneva, I. R. & Weiss, B. (1990). soxR, a locus governing a superoxide response regulon in Escherichia coli K-12. *J Bacteriol* **172**, 4197-4205.

Tsui, H. C., Leung, H. C. & Winkler, M. E. (1994). Characterization of broadly pleiotropic phenotypes caused by an hfq insertion mutation in Escherichia coli K-12. *Mol Microbiol* **13**, 35-49.

Udekwu, K. I., Darfeuille, F., Vogel, J., Reimegard, J., Holmqvist, E. & Wagner, E. G. (2005). Hfq-dependent regulation of OmpA synthesis is mediated by an antisense RNA. *Genes Dev* **19**, 2355-2366.

Udekwu, K. I. & Wagner, E. G. (2007). Sigma E controls biogenesis of the antisense RNA MicA. *Nucleic Acids Res* **35**, 1279-1288.

Ulett, G. C., Webb, R. I. & Schembri, M. A. (2006). Antigen-43-mediated autoaggregation impairs motility in Escherichia coli. *Microbiology* **152**, 2101-2110.

Ulett, G. C., Valle, J., Beloin, C., Sherlock, O., Ghigo, J. M. & Schembri, M. A. (2007). Functional analysis of antigen 43 in uropathogenic Escherichia coli reveals a role in long-term persistence in the urinary tract. *Infect Immun* **75**, 3233-3244.

Urban, J. H. & Vogel, J. (2007). Translational control and target recognition by Escherichia coli small RNAs in vivo. *Nucleic Acids Res* **35**, 1018-1037.

Urbanowski, M. L., Stauffer, L. T. & Stauffer, G. V. (2000). The gcvB gene encodes a small untranslated RNA involved in expression of the dipeptide and oligopeptide transport systems in Escherichia coli. *Mol Microbiol* **37**, 856-868.

van der Woude, M. W. (2006). Re-examining the role and random nature of phase variation. *FEMS Microbiol Lett* **254**, 190-197.

van der Woude, M. W. & Henderson, I. R. (2008). Regulation and function of Ag43 (flu). *Annu Rev Microbiol* **62**, 153-169.

Vanderpool, C. K. & Gottesman, S. (2004). Involvement of a novel transcriptional activator and small RNA in post-transcriptional regulation of the glucose phosphoenolpyruvate phosphotransferase system. *Mol Microbiol* **54**, 1076-1089.

Vecerek, B., Moll, I. & Blasi, U. (2007). Control of Fur synthesis by the non-coding RNA RyhB and iron-responsive decoding. *EMBO J* **26**, 965-975.

Viegas, S. C., Pfeiffer, V., Sittka, A., Silva, I. J., Vogel, J. & Arraiano, C. M. (2007). Characterization of the role of ribonucleases in Salmonella small RNA decay. *Nucleic Acids Res* **35**, 7651-7664.

Viegas, S. C., Silva, I. J., Saramago, M., Domingues, S. & Arraiano, C. M. (2011). Regulation of the small regulatory RNA MicA by ribonuclease III: a target-dependent pathway. *Nucleic Acids Res* **39**, 2918-2930.

Vlassov, A. V., Kazakov, S. A., Johnston, B. H. & Landweber, L. F. (2005). The RNA world on ice: a new scenario for the emergence of RNA information. *J Mol Evol* **61**, 264-273.

Vogel, J., Bartels, V., Tang, T. H., Churakov, G., Slagter-Jager, J. G., Huttenhofer, A. & Wagner, E. G. (2003). RNomics in Escherichia coli detects new sRNA species and indicates parallel transcriptional output in bacteria. *Nucleic Acids Res* **31**, 6435-6443.

Vogel, J., Argaman, L., Wagner, E. G. & Altuvia, S. (2004). The small RNA IstR inhibits synthesis of an SOS-induced toxic peptide. *Curr Biol* **14**, 2271-2276.

Vogel, J. & Wagner, E. G. (2007). Target identification of small noncoding RNAs in bacteria. *Curr Opin Microbiol* **10**, 262-270.

Vogel, J. (2009). A rough guide to the non-coding RNA world of Salmonella. *Mol Microbiol* **71**, 1-11.

Vogel, J. & Luisi, B. F. (2011). Hfq and its constellation of RNA. *Nat Rev Microbiol* **9**, 578-589.

Wagner, E. G. & Brantl, S. (1998). Kissing and RNA stability in antisense control of plasmid replication. *Trends Biochem Sci* **23**, 451-454.

Walkup, L. K. & Kogoma, T. (1989). Escherichia coli proteins inducible by oxidative stress mediated by the superoxide radical. *J Bacteriol* **171**, 1476-1484.

Walter, P. & Blobel, G. (1980). Purification of a membrane-associated protein complex required for protein translocation across the endoplasmic reticulum. *Proc Natl Acad Sci U S A* **77**, 7112-7116.

Walter, P., Ibrahimi, I. & Blobel, G. (1981). Translocation of proteins across the endoplasmic reticulum. I. Signal recognition protein (SRP) binds to in-vitro-assembled polysomes synthesizing secretory protein. *J Cell Biol* **91**, 545-550.

Wang, X., Kim, Y., Ma, Q., Hong, S. H., Pokusaeva, K., Sturino, J. M. & Wood, T. K. (2010). Cryptic prophages help bacteria cope with adverse environments. *Nat Commun* **1**, 147.

Wang, Z., Gerstein, M. & Snyder, M. (2009). RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet* **10**, 57-63.

Washietl, S., Hofacker, I. L. & Stadler, P. F. (2005). Fast and reliable prediction of noncoding RNAs. *Proc Natl Acad Sci U S A* **102**, 2454-2459.

Wassarman, K. M., Zhang, A. & Storz, G. (1999). Small RNAs in Escherichia coli. *Trends Microbiol* **7**, 37-45.

Wassarman, K. M., Repoila, F., Rosenow, C., Storz, G. & Gottesman, S. (2001). Identification of novel small RNAs using comparative genomics and microarrays. *Genes Dev* **15**, 1637-1651.

Waters, L. S. & Storz, G. (2009). Regulatory RNAs in bacteria. *Cell* **136**, 615-628.

Weilbacher, T., Suzuki, K., Dubey, A. K. & other authors (2003). A novel sRNA component of the carbon storage regulatory system of Escherichia coli. *Mol Microbiol* **48**, 657-670.

Weinberg, Z., Barrick, J. E., Yao, Z. & other authors (2007). Identification of 22 candidate structured RNAs in bacteria using the CMfinder comparative genomics pipeline. *Nucleic Acids Res* **35**, 4809-4819.

Wilderman, P. J., Sowa, N. A., FitzGerald, D. J., FitzGerald, P. C., Gottesman, S., Ochsner, U. A. & Vasil, M. L. (2004). Identification of tandem duplicate regulatory small RNAs in Pseudomonas aeruginosa involved in iron homeostasis. *Proc Natl Acad Sci U S A* **101**, 9792-9797.

Wimberly, B. T., Brodersen, D. E., Clemons, W. M., Jr., Morgan-Warren, R. J., Carter, A. P., Vonrhein, C., Hartsch, T. & Ramakrishnan, V. (2000). Structure of the 30S ribosomal subunit. *Nature* **407**, 327-339.

Windbichler, N., von Pelchrzim, F., Mayer, O., Csaszar, E. & Schroeder, R. (2008). Isolation of small RNA-binding proteins from E. coli: evidence for frequent interaction of RNAs with RNA polymerase. *RNA Biol* **5**, 30-40.

Winkler, W., Nahvi, A. & Breaker, R. R. (2002a). Thiamine derivatives bind messenger RNAs directly to regulate bacterial gene expression. *Nature* **419**, 952-956.

Winkler, W. C., Cohen-Chalamish, S. & Breaker, R. R. (2002b). An mRNA structure that controls gene expression by binding FMN. *Proc Natl Acad Sci U S A* **99**, 15908-15913.

Winkler, W. C., Nahvi, A., Sudarsan, N., Barrick, J. E. & Breaker, R. R. (2003). An mRNA structure that controls gene expression by binding S-adenosylmethionine. *Nat Struct Biol* **10**, 701-707.

Wosten, M. M. (1998). Eubacterial sigma-factors. *FEMS Microbiol Rev* **22**, 127-150.

Wu, J. & Weiss, B. (1991). Two divergently transcribed genes, soxR and soxS, control a superoxide response regulon of Escherichia coli. *J Bacteriol* **173**, 2864-2871.

Wu, J. & Weiss, B. (1992). Two-stage induction of the soxRS (superoxide response) regulon of Escherichia coli. *J Bacteriol* **174**, 3915-3920.

Yanofsky, C. (2000). Transcription attenuation: once viewed as a novel regulatory strategy. *J Bacteriol* **182**, 1-8.

Ying, X., Cao, Y., Wu, J., Liu, Q., Cha, L. & Li, W. (2011). sTarPicker: a method for efficient prediction of bacterial sRNA targets based on a two-step model for hybridization. *PLoS One* **6**, e22705.

Yoshihisa, T. (2006). tRNA, new aspects in intracellular dynamics. *Cell Mol Life Sci* **63**, 1813-1818.

Yu, D., Ellis, H. M., Lee, E. C., Jenkins, N. A., Copeland, N. G. & Court, D. L. (2000). An efficient recombination system for chromosome engineering in Escherichia coli. *Proc Natl Acad Sci U S A* **97**, 5978-5983.

Zhang, A., Altuvia, S. & Storz, G. (1997). The novel oxyS RNA regulates expression of the sigma s subunit of Escherichia coli RNA polymerase. *Nucleic Acids Symp Ser*, 27-28.

Zhang, A., Altuvia, S., Tiwari, A., Argaman, L., Hengge-Aronis, R. & Storz, G. (1998). The OxyS regulatory RNA represses rpoS translation and binds the Hfq (HF-I) protein. *EMBO J* **17**, 6061-6068.

Zhang, A., Wassarman, K. M., Rosenow, C., Tjaden, B. C., Storz, G. & Gottesman, S. (2003). Global analysis of small RNA and mRNA targets of Hfq. *Mol Microbiol* **50**, 1111-1124.